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I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

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Dated

29 JUN 1999

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GB9812550.3

By virtue of a direction given under Section 30 of the Patents Act 1977, the application is proceeding in the name of

ENZACTA R&D LIMITED,  
Incorporated in the United Kingdom,  
3rd Floor,  
55 Gower Street,  
LONDON, WC1E 6HQ,  
United Kingdom

[ADP No. 07639552001]

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11 JUN 98 E367340-1 002866  
P01/7700 25.00 - 9812550.3

# Request for grant of a patent

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The Patent Office

Cardiff Road  
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Gwent NP9 1RH

1. Your reference

AEPWAP18966GB

2. Patent application number

(The Patent Office will fill in this part)

9812550.3

3. Full name, address and postcode of the or of each applicant (underline all surnames)

AEPACT Limited

c/o Ronaldsons

3rd Floor, 55 Gower Street

London

WC1E 6HQ

United Kingdom

United Kingdom

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

TUMOUR THERAPY AND IMAGING

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

ERIC POTTER CLARKSON

PARK VIEW HOUSE

58 THE ROPEWALK

NOTTINGHAM

NG1 5DD

Patents ADP number (if you know it)

1305010

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number  
(if you know it)

Date of filing  
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing  
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer yes if:

YES

a) any applicant named in part 3 is not an inventor, or

b) there is an inventor who is not named as an applicant, or

c) any named applicant is a corporate body.

See note (d))

# Patents Form 1/77

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Description	49
Claim(s)	5
Abstract	1
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Translations of priority documents	0
Statement of inventorship and right to grant of a patent (Patents Form 7/77)	NO
Request for preliminary examination and search (Patents Form 9/77)	NO
Request for substantive examination (Patents Form 10/77)	NO
Any other documents (please specify)	

11. I/We request the grant of a patent on the basis of this application.

Signature	Date
ERIC POTTER CLARKSON	11 June 1998

12. Name and daytime telephone number of person to contact in the United Kingdom
- 0115 9552211

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Patents Form 1/77

## TUMOUR THERAPY AND IMAGING

The present invention relates to tumour therapy and imaging, particularly therapeutic systems for activating prodrugs or inactivating rescue agents  
5 and for the use of such systems in killing tumour cells.

The delivery of a cytotoxic agent to the site of tumour cells is much desired because systemic administration of these agents can result in the killing of normal cells within the body as well as the tumour cells. The  
10 resulting toxicity to normal cells limits the dose of the cytotoxic agent and thus reduces the therapeutic potential of these agents. However, in some instances the administered agent has no intrinsic activity but is converted *in vivo* at the appropriate time or place to the active drug. Such analogues are referred to as prodrugs and are used extensively in medicine (Connors  
15 & Knox (1995) *Expert Opinion on Therapeutic Patents* 5, 873-885). Conversion of the prodrug to the active form can take place by a number of mechanisms depending, for example, on changes of pH, oxygen tension, temperature or salt concentration or by spontaneous decomposition of the drug or internal ring opening or cyclisation.  
20

WO 88/07378 describes a two-component system, and therapeutic uses thereof, wherein a first component comprises an antibody fragment capable of binding with a tumour-associated antigen and an enzyme capable of converting a prodrug into a cytotoxic drug, and a second  
25 component which is a prodrug which is capable of conversion to a cytotoxic drug. This general system, which is often referred to as "antibody-directed enzyme prodrug therapy" (ADEPT), is also described

in relation to specific enzymes and prodrugs in EP 0 302 473 and WO 91/11201.

WO 89/10140 describes a modification to the system described in WO 88/07378 wherein a further component is employed in the system. This further component accelerates the clearance of the first component from the blood when the first and second components are administered clinically. The second component is usually an antibody that binds to the antibody-enzyme conjugate and accelerates clearance. An antibody which was directed at the active site on the enzyme had the additional advantage of inactivating the enzyme. However, such an inactivating antibody has the undesirable potential to inactivate enzyme at the tumour sites, but its penetration into tumours was obviated by the addition of galactose residues to the antibody. The galactosylated antibody was rapidly removed from the blood, together with bound antibody-enzyme component, via galactose receptors in the liver. The system has been used safely and effectively in clinical trials. However, galactosylation of such an inactivating antibody which results in its rapid clearance from blood also inhibits its penetration of normal tissue and inactivation of enzyme localised there.

WO 93/13805 describes a system comprising a compound comprising a target cell-specific portion, such as an antibody specific to tumour cell antigens, and an inactivating portion, such as an enzyme, capable of converting a substance which in its native state is able to inhibit the effect of a cytotoxic agent into a substance which has less effect against said cytotoxic agent. The prolonged action of a cytotoxic agent at tumour sites



is therefore possible whilst protecting normal tissues from the effects of the cytotoxic agent.

WO 93/13806 describes a further modification of the ADEPT system comprising a three component kit of parts for use in a method of destroying target cells in a host. The first component comprises a target cell-specific portion and an enzymatically active portion capable of converting a prodrug into a cytotoxic drug; the second component is a prodrug convertible by said enzymatically active portion to the cytotoxic drug; and the third component comprises a portion capable of at least partly restraining the component from leaving the vascular compartment of a host when said component is administered to the vascular compartment, and an inactivating portion capable of converting the cytotoxic drug into a less toxic substance.

Our co-pending patent application PCT/GB97/03284 describes a macromolecule prodrug therapy system (MDEPT) in which the delivery of enzymes to tumour sites can be effected without the use of specific antibodies by taking advantage of the leaking of tumour vessels to macromolecules which combines with the poor lymphatic drainage to allow macromolecules to accumulate at tumour sites.

WO 97/20580 describes the use of enzyme inhibitors in an improvement to ADEPT.

WO 97/24143 describes the use of internalizing antibodies and/or intracellular cofactors in an improvement to ADEPT.

Other tumour therapeutic or diagnostic systems are known which make use of tumour-targeting systems, such as systems which use tumour-selective antibodies which are coupled to biotin, and streptavidin (or avidin) to clear the biotinylated antibody from the blood. For example,

5 Paganelli *et al* (1990) *Int. J. Cancer* 45, 1184-1189 describes the intraperitoneal radiolocalization of tumours pre-treated by biotinylated monoclonal antibodies and Rosebrough & Hashmi (1996) *J. Pharmacol. Exp. Ther.* 276, 770-775 describes galactose-modified streptavidin-GC4 antifibrin monoclonal antibody conjugates and their application for two-

10 step thrombus/embolus imaging. The avidin or streptavidin accelerates clearance of radiolabel from normal tissues.

Still further tumour therapeutic systems make use of toxins which are directed at the target cell or tumour. Typical such systems include

15 antibody-toxin conjugates (immunotoxins).

Although it is now possible to make human antibodies directed at tumour associated antigens by various techniques, such as synthetically, or in mice which have a "human" immune system and thus eliminate or minimise a

20 human host antibody response to foreign immunoglobulins the issue with regard to enzymes which are used for prodrug activation has been more complex. As discussed in WO 89/10140, non-mammalian or xenogeneic enzymes can confer the advantage of specificity but have the disadvantage of being highly immunogenic to the host. The development of host

25 antibodies to an administered enzyme conjugate precludes further use of the conjugate since the host antibodies effect its rapid removal from blood and prevent tumour localisation of the enzyme. Syngeneic enzymes have the potential advantage of being non-immunogenic when conjugated to

syngeneic antibodies but the endogenous distribution of the enzyme in normal body tissues can result in activation of an appropriate prodrug at unwanted sites in normal tissues. Therefore, although the use of syngeneic enzymes may prevent an undesirable immune response, it may not be desirable for other reasons.

The host antibody response can be delayed by administration of immunosuppressive agents such as cyclosporin and we have reported that in patients up to 3 weekly cycles of treatment have been given with cyclosporin (Bagshawe *et al* (1995) *Tumor Targeting* 1, 1-17) and that of eight evaluable patients, four gave a partial response and one gave a mixed response. The cyclosporin allowed three cycles of treatment to be given during a 21 day period. The maximum period of delay in production of detectable levels of host anti-enzyme antibody was 22 days using cyclosporin. However, immunosuppressive agents are toxic and in the presence of cytotoxic agents control of their concentration in blood has proved difficult.

US 5,447,722 to Sehon relates to a method for the suppression of an immune response with antigen-monomethoxypolyethylene glycol (mPEG) conjugates in sensitized individuals. There is no suggestion that suppression of an immune response would be useful in multicomponent cancer therapeutic systems or in the use of immunotoxins.

There remains the need to improve ADEPT and MDEPT systems, and to improve other cancer therapeutic systems, especially multicomponent systems, in which the administration of protein-based components, particularly foreign protein-based components, may lead to unacceptable

immune reactions which, at worst themselves may be life-threatening (for example, by causing anaphylactic shock) or, at least, the potential efficacy of the systems is reduced because anti-system antibodies are present in the patient.

5

Objects of the present invention are to provide improved methods of tumour therapy and diagnosis; in particular, the invention includes the provision of improved methods of MDEPT.

- 10 A first aspect of the invention provides a method of combating or diagnosing a tumour in a patient, the method comprising administering to the patient (a) an agent which tolerizes the patient to a said tumour selective agent or to an agent which interacts selectively with the said tumour selective agent; (b) a tumour selective agent which comprises a
- 15 polypeptide; and (c) at least one further agent which interacts selectively with the said tumour selective agent.

The term "tumour" is to be understood as referring to all forms of neoplastic cell growth, including tumours of the lung, liver, blood cells,

20 skin, pancreas, stomach, colon, prostate, uterus, breast, lymph glands and bladder. Solid tumours are especially suitable for use with MDEPT systems.

By "tumour selective agent" we include any agent which selectively

25 accumulates at the site of a tumour following administration of the agent to a patient with a tumour. Typically, the tumour selective agent accumulates at the site of a tumour to a greater extent than at a non-tumour site such that, for example at 48 hours after administration and

accumulation at the tumour site, the tumour selective agent is at least two-fold more concentrated in the tumour than in non-tumour tissue. Preferably, the tumour selective agent is at least five-fold more concentrated, more preferably at least ten-fold more concentrated. As is described in more detail in WO 89/10140, at least when the tumour selective agent comprises an enzyme for use in an ADEPT or MDEPT system, it is possible to clear the tumour selective agent from the blood so as to lead to an increased relative concentration in the tumour. WO 89/10140 is incorporated herein by reference. Following clearance, the tumour to non-tumour ratios should exceed 60:1.

In a preferred embodiment of the invention the tumour selective agent is an antibody-enzyme complex for use in an ADEPT system. Suitable antibody-enzyme complexes for use in an ADEPT system are well known in the art and are, for example, described in WO 88/07378; Bagshawe (1987) *Br. J. Cancer* 56, 531-532; Bagshawe *et al* (1988) *Br. J. Cancer* 58, 700-703; and Senter *et al* (1988) *Proc. Natl. Acad. Sci. USA* 85, 4842-4846, all of which are incorporated herein by reference.

In a further preferred embodiment of the invention, the tumour selective agent is an antibody-enzyme complex for use in inactivating a rescue agent. Such antibody-enzyme complexes are described in WO 93/13805, incorporated herein by reference.

In a still further preferred embodiment, the tumour selective agent is an enzyme-macromolecule complex such as described in co-pending patent application PCT/GB97/03284. This patent application describes a macromolecule prodrug therapy system (MDEPT) in which the delivery of

enzymes to tumour sites can be effected without the use of specific antibodies by taking advantage of the leaking of tumour vessels to macromolecules which combines with poor lymphatic drainage to allow macromolecules to accumulate at tumour sites. The macromolecule-enzyme complex may be used in a variation of ADEPT (ie MDEPT) or it may be used for use in inactivating a rescue agent essentially as described in WO 93/13805, incorporated herein by reference, and described in more detail below.

It is preferred if the enzyme for use in a prodrug activation system (such as ADEPT or MDEPT) and prodrug are chosen from the following combinations:

Alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs, aryl sulphatase useful for converting sulphate-containing prodrugs into free drugs, cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anticancer drug 5-fluorouracil, proteases such as Serratia protease, thermolysin, subtilisin, carboxy-peptidases and cathepsins that are useful for converting peptide-containing prodrugs into free drugs, D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents, carbohydrate-enzymes such as  $\beta$ -galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs,  $\beta$ -lactamase useful for converting drugs derivatized with  $\beta$ -lactams into free drugs and penicillin amidases useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups into free drugs.

Other enzymes and prodrugs include hydrolases, amidases, sulphatases, lipases, glucuronidases, phosphatases and carboxypeptidases, and prodrugs be prepared from any of the various classes of anti-tumour compounds for example alkylating agents (nitrogen mustards) including  
 5 cyclophosphamide, bisulphan, chlorambucil and nitrosoureas; intercalating agents including adriamycin and dactinomycin; spindle poisons such as vinca alkaloids; and anti-metabolites including anti-folates, anti-purines, anti-pyrimidines or hydroxyurea.

10 Also included are cyanogenic prodrugs such as amygdalin which produce cyanide upon action with a carbohydrate cleaving enzyme.

It is particularly preferred if the portion capable of converting a prodrug into a cytotoxic drug is a carboxypeptidase, especially carboxypeptidase  
 15 G2. It is also preferred that the prodrug is a nitrogen mustard glutamate, more preferably a benzoic acid nitrogen mustard glutamate as described in WO 88/07378. It is also preferred that the prodrug is a nitrogen mustard glutamate derived from phenol or phenylenediamine mustard as described in WO 94/02450 (inventors P.J. Burke, R.J. Dowell, A.B. Mauger and  
 20 C.J. Springer). It is also preferred that the prodrug is of the self-immolative type as described in WO 95/02420 (inventors C.J. Springer and R. Marais).

The potential advantages in using non-antibody macromolecules as  
 25 tumour-selective agents (ie MDEPT system) are substantial. Firstly, a non-specific macromolecule may be selected that is less immunogenic than, for example, an antibody-enzyme complex. Secondly, a macromolecule may be much less costly to produce than humanised

antitumour antibody. Thirdly, whereas an antibody binds to only a limited range of cancers (antibodies only exist for about 60% of all malignancies), the macromolecule uptake by tumours appears to be a characteristic common to all solid cancers so far examined.

5

Thus, many tumours such as sarcomas for which no selective antibodies have yet been reported may be targeted in this principle.

10 The relatively low differential between tumour and non-tumour tissues with non-specific macromolecules is exploitable only if the level of normal tissue enzyme is inhibited, for example by using a galactosylated anti-enzyme antibody. To get the required amount of enzyme to tumour sites when the enzyme is conjugated to a non-specific macromolecule will require a greater amount of such a conjugate to be administered than  
15 would be the case with a specific antibody-enzyme conjugate, but the lower cost of the former should offset its lower efficiency.

Preferably, the macromolecule used for tumour targeting is hydrophilic and is characterised by being soluble in body fluids and in conventional  
20 fluids for parenteral administration. Suitably, the macromolecule is biodegradable so that systemic accumulation during repeated administration is avoided. Clearly, however, it must not be degraded so fast as to fail to accumulate at the tumour site. Preferably, when conjugated to the selected enzyme, the molecular weight and size of the  
25 conjugate exceeds that of the renal threshold for urinary excretion (MW 60 000), as this helps the blood concentration to be sufficient to provide an effective blood:tumour concentration gradient. A molecular weight of up to at least 800 000 is generally suitable, for example up to 160 000. The



macromolecule is preferably one which is not readily captured by the reticuloendothelial system. To make it catalytic, the macromolecule may be conjugated to one or more enzyme molecules by simple chemical methods, using bi-functional agents which do not degrade the attached  
 5 enzyme. Preferably, the starting macromolecule confers reduced immunogenicity on an immunogenic enzyme to which it is conjugated.

The molecular weights given exclude any water of hydration.

- 10 Macromolecules that are available as sub-units and are not biodegradable may be linked by biodegradable linking units so that the non-biodegradable components are filtered through the kidneys and excreted in the urine.
- 15 Alternatively, it is preferred if the polymer used to make the macromolecule is not biodegradable then the molecular weight of any non-biodegradable portion of the conjugate should be less than the renal threshold (circa 70000) so that after degradation of the biodegradable portion the residual non-biodegradeable portion is excreted through the  
 20 kidneys.

Whereas some macromolecules are not known to be internalised by cells others, such as N-(2-hydroxypropyl)methylacrylamide, are internalised through more than one mechanism (Duncan *et al* (1996) "The role of  
 25 polymer conjugates in the diagnosis and treatment of cancer" in *STP Pharma Sciences* 6, 237-263).

Preferably, the macromolecule is polyethylene glycol (PEG). Derivatisation of proteins with polyethylene glycol has been demonstrated numerous times to increase their blood circulation lifetimes as well as decrease their antigenicity and immunogenicity. A wide variety of methods has been developed to couple PEG to proteins and these are summarised in PCT/GB97/03284, incorporated herein by reference.

Conveniently, the macromolecule may be any of a dextran; a polyamino acid; or a non-tumour-specific protein such as an immunoglobulin, an albumin or a transferrin. Suitably, it may be a copolymer of styrene and maleic anhydride, or may be polyaspartic acid, poly-L-lysine or polyethyleneimine.

The advantage of being able to use a non-antibody macromolecule for targeting an enzyme to tumour sites extends to other applications of tumour located enzymes. Some molecules, in particular some polymers, are known to be readily internalised by tumour cells so that advantage can be taken of enzymes, such as nitroreductase, that require intracellular co-factors, such as NAD(P)H (the reduced form of nicotinamide adenine dinucleotide (phosphate)), to activate prodrugs that are also internalised and convert them to highly potent cytotoxic substances. Such cytotoxic agents are therefore able to kill the cells which internalise the macromolecule enzyme and the prodrug and to release sufficient toxic drug into the extracellular space to achieve a concentration of cytotoxic drug that is lethal to adjacent cells (see US Patent Application No 60/009,361, filed December 29, 1995, incorporated herein by reference which is published as WO 97/24143; PCT/GB96/03254).

Alternatively, catalytic macromolecules (enzyme-macromolecule complexes) can be exploited beneficially in the context of cytotoxic antimetabolite compounds for which normal metabolic components exist and which can be used to block the action of the antimetabolite. In this situation, as is described in detail in WO 93/13805, the catalytic macromolecule (ie macromolecule-enzyme conjugate) can be used to degrade a tumour-protective metabolite at tumour sites whilst allowing the metabolite to protect the normal tissues. In this instance the macromolecule enzyme conjugate is first administered and allowed time to localise at tumour sites. An anti-enzyme antibody is administered say 24 hr after the enzyme conjugate and has the effect of reducing enzyme activity in the blood and normal tissues so that the subsequently co-administered antimetabolite and metabolite result in the metabolite protecting normal tissues whereas, in the tumour tissue, persisting enzyme inactivates the metabolite and thus exposes the tumour cells to the action of the antimetabolite. See WO 93/13805; in particular Figure 2 of WO 93/13805 illustrates the system in relation to CPG2 directed to the target cell and being used to remove folinic acid from the tumour site so that there is no longer inhibition of the action of trimetrexate (incorporated herein by reference).

Hence, any sufficiently non-toxic substance which in its native state is able to inhibit the effect of a cytotoxic agent may be converted into a substance which has less effect on said cytotoxic agent. A suitable compound is folinic acid. Folinic acid reverses the biological effect of the cytotoxic agent trimetrexate, for example, which acts on the enzyme dihydrofolate reductase. Folinic acid is deglutamated and rendered inactive against

trimetrexate by the enzyme carboxypeptidase G2 and other deglutamating enzymes.

Further examples are given in WO 93/13805, incorporated herein by  
5 reference.

It will be appreciated that, as is described in WO 89/10140 (incorporated herein by reference), it is preferred that a further component accelerates the clearance of the antibody-enzyme complex or enzyme-macromolecule  
10 complex from the blood. The further component is typically an antibody that binds to the antibody-enzyme complex or enzyme-macromolecule complex and accelerates clearance.

The action of the antibody-enzyme complex or enzyme-macromolecule  
15 complex can, in a further preferred embodiment, be inhibited by a small enzyme inhibitor as is described in WO 97/20580, incorporated herein by reference.

In a further embodiment of the invention, the tumour selective agent is an  
20 antibody-biotin complex and the at least one further agent which interacts selectively with the said tumour selective agent comprises avidin or streptavidin. For example, suitable systems are described in Paganelli *et al* (1990) *Int. J. Cancer* 45, 1184-1189 and Rosebrough & Hashmi (1996) *J. Pharmacol. Exp. Ther.* 276, 770-775. The tumour selective agent (eg  
25 antibody-biotin conjugate) may be detectably labelled such that following localization to a tumour the localization can be detected. Suitable detectable labels include radiolabels which are well known in the art.

In all of these systems the patient, upon administration of the tumour selective agent which comprises a polypeptide, or upon administration, of a polypeptide-containing further agent which interacts selectively with the said tumour selective agent (such as avidin or streptavidin which binds to an antibody-biotin complex), is likely to develop an immune response to the polypeptide-containing component.

For example, administration of antibody-enzyme conjugates, in particular wherein the antibody or enzyme or both are from a different species to the patient, leads to an immune response.

Similarly, although to a lesser extent than for antibody-enzyme conjugates, a macromolecule-enzyme complex (at least those which accumulate at a tumour and are described in PCT/GB97/03284) may give rise to an immune response. For example, in relation to an MDEPT system using carboxypeptidase (CPG2), a macromolecule-enzyme complex with 43 molecules of mPEG/molecule of CPG2 induced a host response in mice. The response was delayed and the antibody level was lower than that induced by native CPG2 or that induced with a CPG2 complex with 15 to 30 molecules of mPEG/molecule of CPG2. CPG2 with 60 molecules of mPEG/molecule of CPG2 did not evoke an immune response when challenged with native CPG2 or with a CPG2 complex with 20 molecules of mPEG/molecule CPG2.

In addition, administration of streptavidin or avidin to a patient gives rise to an immune response.

The agent which is administered in step (a) is an agent which tolerizes the patient to a said tumour selective agent or to an agent which interacts selectively with the said tumour selective agent.

5 Typically, the tolerizing agent comprises the said tumour selective agent (or said agent which interacts selectively with said tumour selective agent) modified so as to induce tolerance to the tumour selective agent (or agent which interacts selectively with said tumour selective agent) so that the patient, once tolerized with the tolerizing agent, raises substantially no  
10 immune response (or a substantially reduced immune response) when administered the tumour selective agent (or agent which interacts selectively with the tumour selective agent). The reduction or suppression in the immune response which it is desirable to achieve in the practice of the invention is that which will allow sufficient repeated administrations of  
15 the tumour selective agent (or said agent which interacts selectively with said tumour selective agent) in the given therapeutic regimen to achieve a therapeutically beneficial result to the patient.

It is preferred that the agent in step (a) tolerizes the patient to the said  
20 tumour selective agent and comprises said polypeptide wherein immunogenic portions of the polypeptide are masked by a polymer. Thus, typically, when the tumour selective agent is an antibody-enzyme complex epitopes of the antibody-enzyme complex are masked by the presence of a suitable polymer.

25

Suitable polymers for masking epitopes include monomethoxy polyethylene glycol (mPEG), certain dextrans of various molecular weights, hydroxypropylmethyl acrylamide (HPMA), polygalacturonic

acid, N-(2-hydroxypropylmethylacrylamide) copolymers, poly(-L-glutamic acid), poly(hydroxyethyl-L-glutamine), poly( $\alpha$ -malic acid), poly(aspartic acid) PEG copolymers, poly L-lysine, copolymers of poly(ethylmelamine) and palmitic acid, and copolymers of styrene and maleic anhydride. The  
5 polymers which are suitable for the purpose of making a tolerogenic molecule are those that are (i) non-immunogenic themselves (some dextrans are immunogenic and therefore are not suitable) and (ii) are able to mask immunogenic sites (epitopes) in the protein or polypeptide. Other desirable properties of the polymers used for masking epitopes are that  
10 they are hydrophilic, that they are soluble in body fluids and soluble in conventional for parenteral administration.

Where the term "PEG" is used in relation to a specific conjugate, the term means monomethoxy polyethylene glycol (mPEG). Similarly, when the  
15 term "PEGylated" or "PEGylation" is used in relation to a specific complex, this refers to mPEG. Branched chain polymers which are non-antigenic, such as those described in US Patent No 5,643,575, incorporated herein by reference, may also be useful. Methods for attaching such polymers onto an enzyme are well known in the art.  
20 Methods of attaching PEG to a protein are disclosed in Table 1 and the references attached thereto. The conjugation of PEG or dextran to enzymes is described in WO 90/13540, incorporated herein by reference.

We believe that it is particularly useful to mask highly immunogenic  
25 portions of the antibody-enzyme complex in order to get maximum tolerance. In the case where the antibody is human or humanized but the enzyme is of a non-human origin (and the patient is a human) it is

particularly useful to mask the immunogenic sites on the enzyme whether or not any sites in the antibody are masked.

5 The masking of the epitopes of any of the polypeptide-containing components as herein disclosed may be achieved by reacting the polypeptide with a suitable activated form of the polymer such that once the polymer is attached to the polypeptide, the epitopes are masked. Masking may be achieved by the activated polymer reacting with sites which form part of the epitope, or it may be achieved by the polymer  
10 reacting with sites adjacent to the epitope but the polymer nevertheless masking the epitopes. Suitable systems for masking epitopes with polymers in order to form tolerogenic molecules are described in US 5,447,722 to Schon, incorporated herein by reference.

15 In relation to macromolecule-enzyme complexes, PCT/GB97/03284 notes that certain macromolecule-enzyme complexes may be non-immunogenic but it does not disclose or suggest that a two-stage system would be useful, particularly for macromolecule-enzyme complexes which may be immunogenic, particularly upon repeated administration.

20 By "at least one further agent which interacts selectively with the said tumour selective agent" we include various further agents depending on the particular system.

25 For example, with ADEPT and MDEPT systems, the further agent is a prodrug which can be converted into a cytotoxic drug by the enzyme component of the tumour selective agent. Of course, as is described



above, a still further agent which may be used in the system is a clearing antibody or an inhibitor of the enzyme.

As a further example, with the rescue agent inactivation system described  
5 in WO 93/13805, the further agent is the rescue agent which is specifically inactivated by the enzyme component of the tumour selective agent. Of course, as is described above, a still further agent which may be used in this system is a clearing antibody or an inhibitor of the enzyme.

10 As a still further example, in the system as disclosed above which uses a biotinylated tumour-selective antibody, the further agent comprises streptavidin or avidin (by which we include any biotin-binding portion or variant of streptavidin or avidin). Thus, in this system the tolerogenic agent is a suitably-modified form of streptavidin or avidin which induces  
15 immune tolerance (such as PEGylated streptavidin), the tumour selective agent which comprises a polypeptide is biotinylated tumour-selective antibody, and the at least one further agent which interacts selectively with the said tumour selective agent is streptavidin or avidin.

20 In the course of studies relating to MDEPT using macromolecules to deliver enzymes to tumour sites we have found (1) that a polymer enzyme conjugate localises in tumour tissue and (2) that *prior* administration of a polymer-enzyme conjugate can induce a state of immune tolerance in which there is no detectable host antibody response to the foreign protein.  
25 The reduction of immunogenicity by such conjugation has been reported previously with respect to other proteins but our findings indicate that an enzyme may be modified by appropriate conjugation so as to facilitate its delivery to tumour sites through the EPR effect (enhanced penetration and

retention of macromolecules) and to avoid immunogenicity. The polymer-enzyme conjugates optimal for the two functions are not necessarily identical.

5 By immune tolerance we mean a state in which the tolerised host substantially fails to mount an antibody response to the native antigen. In the context of this invention, the native antigen is the tumour selective agent which comprises a polypeptide, or is the agent which interacts selectively with said tumour selective agent.

10

We have found that a polymer enzyme conjugate can be made such that it localises at tumour sites in nude mice xenografted with human tumours. Such conjugates have prolonged half-life in blood and in order to be useful for therapeutic purposes it is necessary to clear the conjugate from blood  
15 and normal tissues without removing much enzyme from tumour sites. We have shown that administration of an antibody directed at a polymer-enzyme conjugate capable of localising at tumour sites can result in accelerated clearance of the antibody-polymer enzyme complex from the blood (see PCT/GB97/03284). The antibody we have used for this is  
20 typically directed at the active site of the enzyme and is galactosylated to achieve its rapid removal from blood *via* hepatic galactose receptors together with enzyme conjugate bound to it. The enzyme is rapidly degraded by the hepatic cells. The galactosylation and rapid removal from blood minimises the effect of the antibody on tumour located enzyme.  
25 Antibodies directed at any part of the polymer enzyme conjugate can be used for this purpose.

In relation to the enzyme carboxypeptidaseG2 (CPG2), the optimum ratio of mPEG molecules to CPG2 for tumour localising function is in the range 5-35 and varies with the molecular weight of mPEG that is used.

- 5 The number of mPEG molecules that can be attached to an enzyme is variable according to the size of the enzyme, the number of ligand groups and the reaction conditions. We have found that bacterial enzyme carboxypeptidase G2 is highly immunogenic in both human and murine recipients with anti-CPG2 antibodies appearing 9-10 days after
- 10 administration. Polymer-enzyme conjugates in which mPEG/CPG2 ratio is  $>50$  have been administered to normal mice according to various protocols and followed without antibodies being detected in the blood of some recipients subsequently. Moreover, when mice which failed to respond to PEG-CPG2 were challenged with native CPG2 they failed to
- 15 generate detectable antibodies. This observation suggested that immunological tolerance had been induced and this was confirmed by transfer of spleen cells from a tolerant mouse to a normal non-immunised mouse which was then challenged with native CPG2 but failed to respond.
- 20 mPEG-CPG2 with a ratio mPEG:CPG2  $>50$  has a prolonged half life in the blood of mice, and is expected to behave similarly in man. Although such conjugates retained a high proportion of their catalytic function they were less readily cleared from blood by the anti-enzyme antibody, presumably because the antibody could not access its binding site through
- 25 the presence of the mPEG.

From the foregoing, it will be appreciated that polymer enzyme conjugates that induce tolerance are likely to localize in tumours but not necessarily

as efficiently as polymer-enzyme conjugates which have been optimized for tumour localization (and which are likely to contain less polymer per enzyme than the complexes which are optimal for tolerization). Thus, a two step approach for MDEPT in which a tolerizing agent is administered to achieve tolerization to a subsequently administered molecule which is optimised for tumour localization is provided by the invention.

Tumour blood vessel fenestration is variable within and between tumours. Some gaps admit molecules up to 400-600 nm in diameter, but other gaps only admit much smaller molecules (see, for example, Yuan *et al* (1995) *Cancer Res.* 55, 3572-3576 entitled "Vascular permeability in a human tumour xenograft: molecular size and cut off size"). Polymer enzyme conjugates optimised for their tumour localizing efficiency are likely to be immunogenic but it is possible that a conjugate optimised for tumour localizing efficiency is also tolerogenic.

In any event, in relation to the practice of the invention where an MDEPT system is used, the skilled person can readily determine the level of polymer to be attached to an enzyme in order to provide an enzyme polymer complex that leads to tolerization upon administration, and the skilled person can readily determine the level of polymer to be attached to an enzyme in order to provide an enzyme polymer complex that accumulates in tumours. In relation to the enzyme CPG2, and as described below, the level of PEGylation for tumour localization is, preferably, within the range 10-35 mPEG molecules per CPG2 molecule. CPG2 has a  $M_r$  of 83 000. For tolerogenesis, the optimum level of PEGylation is in excess of 50 PEG molecules per CPG2 molecule using mPEG of  $M_r$  5000, for example 60 PEG molecules.

A polymer enzyme conjugate may be cleared from the non-tumour (eg blood) compartment using any suitable technique. Preferably, in order to allow clearance of the enzyme-polymer conjugate (whether or not the tolerizing conjugate or the tumour-localizing conjugate) an antibody is used wherein the antibody binds selectively to the enzyme and that the polymer does not sterically hinder access by the antibody to its binding site. Site-directed control of polymer conjugation could be used to ensure that the polymer does not obscure the antibody binding site but it is preferred that the number of polymer molecules/molecule of enzyme allows for binding of the clearance antibody (while still allowing for the enzyme conjugate to be tolerogenic or able to localize in the tumour).

Clearance with an antibody directed at an epitope on the enzyme (or in case of antibody enzyme conjugates on the antibody) requires access for the antibody's binding site to the epitope. If this site is masked by the polymer, attachment of the clearing antibody cannot occur.

Small enzyme inhibitors may gain access to the enzyme active site when it is blocked to the larger clearing antibodies, and may therefore be used.

Alternatively, and as described in WO 89/10140, and in Sharma *et al* (*Br. J. Cancer* 61, 659-662 (1991) "Inactivation and clearance of an anti-CEA CPG2 conjugate in blood after localisation in a xenograft model") the enzyme conjugate may be galactosylated and hepatic galactose receptors blocked by an appropriate molecule. Appropriate molecules for this purpose (in the mouse) are asialofetuin and bovine submaxillary gland mucoprotein, and suitable molecules are believed to be available for

human purposes. Such blocking molecules can preferentially block hepatic galactose receptors allowing the galactosylated PEGylated-enzyme conjugate to accumulate at tumour sites. After a suitable interval - say 4-24 hours - the galactose receptor blockade is terminated and the galactose receptors then remove the galactosylated enzyme conjugate.

Galactosylated HPMA is believed to be suitable for the blockade function demonstrated with asialofetuin and bovine submaxillary gland mucoprotein in the mouse.

10

Anti-polymer antibodies may also be used for clearing.

15

Clearing antibodies are preferably human for human patients or if non-human in origin could be the subject of a tolerisation process themselves.

20

The method of using these agents to localise an enzyme at tumour sites such that there is no host antibody response is illustrated as follows in relation to PEG:CPG2 conjugates. The skilled person can readily adapt and devise methodology for the manufacture and administration of the tolerizing agent and the subsequent agent. Since the induction of tolerance with a polymer-enzyme conjugate or antibody-enzyme conjugate is an active immunological response, it is preferred that the time between the administration of the tolerizing agent and the subsequent agent is not less than the time required to induce a primary immune response. Typically, this time is about 7 to 10 days, and so the interval between tolerising and tumour localizing doses of polymer-enzyme conjugate or other conjugates is greater than 7 days, more preferably greater than 10 days.

25

(1) Administration by parenteral route of an enzyme conjugated to mPEG (the tolerogenic conjugate); the average mPEG:CPG2 ratio is >50. CPG2 has a molecular weight of 83000 Da; the number of molecules of mPEG required per molecule for other enzymes will be determined by the number of available lysine groups and will be broadly proportionate to the surface area of the molecule. The actual number needs to be ascertained on an individual basis. Such administration may be repeated after an interval of several days. The amount of tolerogenic mPEG CPG2 per immunisation in the mouse is in the range 10-50 µg and is likely to be in the 0.5-5 mg in the human.

(2) Administration of CPG2 conjugated to mPEG such that the mPEG:CPG2 ratio is in the range 5-35 mPEG molecules per molecule of CPG2, (the tumour localising molecule). For other enzymes the ratio will be similar but each enzyme will require optimisation with respect to (1) Peak concentration at tumour sites (2) Retention at tumour sites (3) Amount of clearance antibody required to remove residual enzyme from blood 12-36 hours after administration. Administration of the tumour localising PEG-enzyme conjugate may be delayed until clearance of the tolerogenic molecule has been effected.

The system is applicable to other non-human enzymes such as  $\beta$ -glucuronidase, nitroreductase and  $\beta$ -lactamase. In the case of less potent immunogens than carboxypeptidase it is possible that a level of PEGylation can be found which is tolerogenic and yet undergoes clearance effectively with an appropriate clearance system.

Clearance systems for clearing tolerogenic molecules include antibodies directed at the enzyme component of the tolerizing enzyme-polymer complex as described above.

- 5 Allowing 7-14 days after administering the tolerogenic conjugate and a tumour localizing conjugate provides for its progressive clearance from blood and tissues and only low levels of enzyme remain at 14 days. Anti-polymer antibodies and small enzyme inhibitors may be used if the clearance anti-enzyme antibody proves ineffective.

10

- As is noted above, the system is applicable also to antibody-enzyme conjugates used for tumour targeting, such as A5B7-CPG2 and A5B7 (Fab')<sub>2</sub>-CPG2 which are mentioned in WO 89/10140 and other antibodies conjugated to CPG2. The antibody in the conjugate may be human or
- 15 non-human in origin; it may be polyclonal or monoclonal, it may be an intact IgG molecule or a fragment of an antibody; it may be a chemical conjugate or a recombinant molecule such as a single chain Fv fusion protein. When the masking polymer is PEG, the tolerogenic compound will have a PEG:Ab-enzyme ratio such that substantially all immunogenic
- 20 sites on the conjugate are masked by the polymer. Typically, in relation to any polypeptide, immunogenic sites contain lysine and since, typically, it is a lysine residue which is reacted with (and blocked by) the polymer, the immunogenic site is blocked. However, for immunogenic sites that do not contain lysine, the immunogenic site may still be masked by polymer
- 25 attached to lysine residues at adjacent sites. Alternatively, the polymer may be attached to the enzyme by non-lysine residues. The tolerogenic immunisation schedule with the mPEG-mAb enzyme is similar to that for the mPEG-enzyme.



Following the induction of immune tolerance to the antibody enzyme conjugate the unmodified enzyme conjugate may be administered repeatedly and as necessary for therapeutic purposes.

5

A particularly preferred embodiment is a therapeutic system comprising (i) a maximally PEGylated conjugate comprising a tumour selective antibody, such as A5B7 or a fragment thereof, conjugated to an enzyme, such as carboxypeptidase G2, for prior administration to induce immune tolerance  
 10 to subsequently administered conjugates incorporating the same proteins; (ii) after an interval of several days to allow clearance of the above conjugate there is administered an antibody-enzyme conjugate which may or may not be conjugated to mPEG but if conjugated to mPEG the ratio of mPEG:antibody enzyme is optimised (a) with respect to tumour  
 15 localisation and (b) with respect to its accelerated clearance by means of administering; (iii) a further component which is a clearing antibody directed at the enzyme or antibody; (iv) after enzyme activity in blood, and by implication, in normal tissues has fallen to below the level of detection by a sensitive method (spectroscopy or HPLC) a prodrug is  
 20 administered, the prodrug is a substrate for the enzyme and the enzymatic action results in the generation of a cytotoxic agent. Alternatively a cytotoxic drug and its rescue agent are co-administered and the rescue agent is a substrate for the targeted enzyme.

25 A further aspect of the invention provides use of an agent which tolerizes a patient to a tumour selective agent in the manufacture of an agent for combating or diagnosing a tumour in a patient wherein said tumour selective agent comprises a polypeptide and wherein said patient is

administered said tolerizing agent prior to administration of said tumour selective agent and prior to administration of a further agent which interacts selectively with said tumour selective agent.

- 5 A still further aspect of the invention provides a polymer-enzyme conjugate wherein substantially all immunogenic sites of the enzyme are masked by the polymer and wherein the enzyme is able to convert a substantially inactive prodrug into a cytotoxic drug, or wherein the enzyme is able to inactivate a rescue agent.

10

The polymer-enzyme conjugate may contain any of the polymers herein described as being useful in imparting tolerogenicity on the enzyme. The enzyme may be any suitable enzyme for use in activating a prodrug into a cytotoxic drug. Suitable enzymes are described herein. It is preferred if  
15 the enzyme is any one of a hydrolase, amidase, sulphatase, lipase, glucuronidase, phosphatase or carboxypeptidase.

It is particularly preferred if the enzyme is carboxypeptidase G2. It is particularly preferred if the polymer is mPEG.

20

As is herein disclosed CPG2-mPEG conjugates consisting of at least 50 mPEG molecules per molecule of CPG2 evoke immune tolerance to CPG2.

- 25 A still further aspect of the invention provides an antibody-enzyme-polymer conjugate wherein substantially all immunogenic sites of the enzyme are masked by the polymer and wherein the enzyme is able to

convert a substantially inactive prodrug into a cytotoxic drug, or wherein the enzyme is able to inactivate a rescue agent.

In a preferred embodiment substantially all immunogenic sites of the antibody of the antibody-enzyme conjugate are also masked by the polymer.

A still further aspect of the invention provides a method of destroying a target cell in a patient the method comprising administering to the patient (a) an agent which tolerizes the patient to a said target cell selective agent and (b) a target cell selective agent which comprises a target cell selective portion and an agent capable of killing the target cell.

It is particularly preferred that the agent capable of killing the target cell is a toxin such as diphtheria toxin (see Moolten & Cooperbrand (1970) *Science* 169, 68- ), ricin A (Press *et al* (1986) *Cell Immunol.* 102, 10-20), abrin (Olsnes *et al* (1974) *Nature* 249, 627-631), gelonin (Thorpe *et al* (1981) *Eur. J. Biochem.* 116, 447-454), *Pseudomonas* exotoxin (Pirker *et al* (1985) *Cancer Res.* 45, 751-757), immunotoxin (Vitteta *et al* (1983) *Science* 219, 644-650), OK-432, a streptococcal agent (Kimura *et al* (1979) *Acta Med. Okayama* 33, 471-478). See also Pastern (1998) Abstracts for Advances in the Application of Monoclonal Antibodies, Santorini, Greece, May 1998.

The tolerogenic agent typically consists of the antibody-toxin which has had substantially all its epitopes masked with a suitable polymer as herein disclosed.

A yet still further aspect provides a therapeutic system for treating a patient comprising (a) an agent which tolerizes the patient to a said target cell selective agent and (b) a target cell selective agent which comprises a target cell selective portion and an agent capable of killing the target cell.

5

Further aspects provide use of an agent which tolerizes a patient to a target cell selective agent in the manufacture of a medicament for destroying a target cell in the patient wherein the patient is administered said tolerizing agent prior to administration of said target cell selective agent and a polymer-polypeptide conjugate wherein substantially all immunogenic sites of the polypeptide are masked by the polymer and wherein the polypeptide comprises a target cell selective portion and a portion capable of selectively killing the target cell.

15 The target cell specific portion is preferably an antibody which selectively binds to a target cell. Suitable target cell selective antibodies include antibodies which selectively bind to a tumour associated antigen as is well known in the art.

20 The skilled person will readily appreciate that similar considerations relating to the type of polymers that evoke immune tolerance and the timing of administration of a tolerogenic molecule compared to the target cell selective molecule for, for example, antibody-toxin conjugates, apply as for the antibody-enzyme conjugates disclosed herein.

25

It will be appreciated that the components of the therapeutic systems herein described are administered as suitable pharmaceutical compositions

or formulations wherein the component is in admixture with a pharmaceutically compatible carrier.

Formulations suitable for parenteral administration (which is a suitable route of administration) include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use.

The invention also provides components of the therapeutic systems herein disclosed packaged and presented for use in medicine.

The invention will now be described in more detail with reference to the following Examples and Figures wherein

Fig 1 shows the superimposed gel filtration chromatogram of purified PEG-CPG2 and native CPG2 determined separately using a Pharmacia Superose 6HR 10/12 FPLC column (—) PEG-CPG2), (---) native CPG2.

Fig 2 shows the molecular weight standard curve for a Superose 6HR gel filtration column.

Fig 3 shows isoelectric focusing of EEA/008/16 and native CPG<sub>2</sub> on ampholine PAGplate pH3.5-9.5 range.

Wells nos. 1, 2 and 3 show focused protein bands for isoelectric point (pI) calibration markers, native CPG<sub>2</sub> and EEA/008/116 respectively. Native CPG<sub>2</sub> was resolved into 3 bands which represent 3 different isoforms. EEA/008/116 was resolved into 2 focused bands which represent 2 different charged species of PEG modified CPG<sub>2</sub>.

Fig 4 shows the determination of the isoelectric point of EEA/008/116 (CPG<sub>2</sub>-PEG 5000) and native CPG<sub>2</sub> using isoelectric focusing using pI marker calibration curve of amphline PAGplate pH3.5-9.5 range.

The position of the 3 focused bands of native CPG<sub>2</sub> corresponds to pIs of 6.4, 6.6, and 6.7 whilst the position of the 2 EEA/008/116 bands corresponds to pIs of 5.28 and 5.02. PEG reacts with the primary amino group of lysine residues in proteins. Lysine residues that are coupled to PEG cannot be protonated and hence each PEG molecule attached to CPG<sub>2</sub> result in the loss of a positive charge on the surface of the protein. The loss of positive charge gives rise to a shift in the pI of the protein towards acidic pH.

Fig 5 shows polyacrylamide gel electrophoresis of EEA/008/116 and native CPG<sub>2</sub> under denaturing, reduced conditions.

25

Wells nos. 3, 4 5 were loaded with molecular weight markers. Well nos. 1 and 2 were loaded with EEA/008/116 native CPG<sub>2</sub> respectively. CPG<sub>2</sub> shows a single band for the monomer of molecular weight 42,000 daltons

whilst EEA/008/116 shows an apparent molecular weight for the monomer of 133,437 daltons.

Fig 6 shows the molecular weight calibration curve established using  
5 HMW and LMW calibration proteins for SDS-PAGE on a 4-20% gradient gel.

Fig 7 shows the biodistribution of F(ab')<sub>2</sub> A5B7-CPG2 conjugate in nude mice bearing LS174T human colon carcinoma xenografts.

10

Fig 8 shows the biodistribution of mPEG-A5B7-CPGS conjugate in nude mice bearing LS174T human colon carcinoma xenografts.

Fig 9 shows the biodistribution of mPEG-CPG2 conjugate in nude mice  
15 bearing LS174T human colon carcinoma xenografts.

Fig 10 shows the immune response to CPG2 preparations in Balb/C mice.

20 **Example 1: Method of conjugation of mPEG to carboxypeptidase G<sub>2</sub>, and *in vivo* distribution and immunogenicity of conjugates**

1.5 g of methoxypolyethylene glycol p-nitrophenyl carbonate (Sigma) of molecular weight 500 was added to 109.99 mg of carboxypeptidase G<sub>2</sub> (CPG<sub>2</sub>, Centre for Applied Microbiological Research, Porton Down,  
25 Wiltshire, UK) in 23.1 ml of 0.2M sodium phosphate, pH 8.08, a method by Veronese *et al* (1985) *Applied Biochemistry, Biotechnology* 11, 141-152. After 2 hours at room temperature unreacted PEG was removed by five successive ultrafiltration steps in an ultrafiltration cell (Amicon) using

an XM-50 dialysis membrane. Each filtration was accomplished using 50 ml of 0.05M sodium phosphate, 0.15M sodium chloride, pH 7.0 (PBS) as the dialysing solution. PEGylated protein was separated from unmodified protein by FPLC gel filtration using a Superose 6HR 10/12 column (Pharmacia) equilibrated in PBS and eluted with the same buffer. Figure 1 shows the gel filtration chromatograms of purified PEG-CPG<sub>2</sub> compared with native CPG<sub>2</sub>. The effective molecular size of PEG-CPG<sub>2</sub> was determined to be 1,260,162 Da. The average number of PEG molecules attached per CPG<sub>2</sub> can be varied by altering the ratio of 'activated PEG' to CPG<sub>2</sub> in the initial conjugation reaction. For example, PEG-CPG<sub>2</sub> conjugates with an average of 60.3 molecules of PEG attached per CPG<sub>2</sub> was prepared by reacting 500 mg of 'activated PEG' with 5 mg of CPG<sub>2</sub>. PEG-CPG<sub>2</sub> conjugates with an average of 21 molecules of PEG attached per CPG<sub>2</sub> was prepared by reacting 80 mg of 'activated PEG' with 10 mg of CPG<sub>2</sub>.

CPG<sub>2</sub> activity was measured spectrophotometrically in 0.1M Trizma-base, 0.1 mM zinc chloride, pH 7.3 containing 0.06 mM methotrexate (final assay volume 1.0 ml). The reaction was initiated by the addition of 10  $\mu$ l (33.12  $\mu$ g) of PEG-CPG<sub>2</sub> and enzyme activity was measured by the decrease in absorbance at 320 nm. One unit of CPG<sub>2</sub> corresponds to 1  $\mu$ mol of methotrexate hydrolysed min<sup>-1</sup> (Sherwood *et al* (1983) "Purification and properties of carboxypeptidase from pseudomonas strain RS16" *Eur. J. Biochem.* 148, 447-453). The specific activity of PEG-CPG<sub>2</sub> was determined to be 243.32 $\pm$ 22.76 U/mg.

The amount of PEG attached to CPG<sub>2</sub> was determined indirectly by quantifying the residual number of unmodified amino groups in the



derivatised protein using trinitrobenzene sulphonic acid (TNBS) according to the method of Habeeb (1966) "Determination of free amino acid groups in proteins by trinitrobenzene sulfonic acid" *Anal. Biochem.* 14, 328-336. 20  $\mu$ l, 40  $\mu$ l, 60  $\mu$ l, 80  $\mu$ l, 100  $\mu$ l and 120  $\mu$ l aliquots of PEG-CPG<sub>2</sub> (1  
 5 mg/ml in 0.05M sodium phosphate, pH 7.2) and the same aliquots of native CPG<sub>2</sub> (1 mg/ml in 0.05 M sodium phosphate, pH 7.2) were assayed by the Habeeb method. From the linear regression curves of the reactions of PEG-CPG<sub>2</sub> and native CPG<sub>2</sub> with TNBS we calculated that the average number of PEG molecules attached per CPG<sub>2</sub> was 42.8.

10

As part of the characterisation of PEG-CPG<sub>2</sub> the isoelectric point (pI), which is an intrinsic physiochemical parameter of a protein, was measured. The pI of a protein is defined under specific conditions where it migrates in a pH gradient generated by an electric field. Under these  
 15 conditions, the protein migrates until it reaches a position in the pH gradient at which its net surface charge, or pI, is zero. PEG-CPG<sub>2</sub> (1 mg/ml in 1% (w/v) glycine) and native CPG<sub>2</sub> (1 mg/ml in 1% (w/v) glycine) were focused on precast Ampholine PAGplates pH range 3.5-9.5 (Pharmacia) using the multiphor II electrophoresis unit (Pharmacia)  
 20 according to the manufacturers instructions for isoelectric focusing (IEF). Broad pI markers (Pharmacia) were focused along with the samples to obtain a calibration curve for the IEF gel. As Figure 3 shows, three charged isoforms of native CPG<sub>2</sub> were observed (pIs of 6.7, 6.6 and 6.4) whilst two focused bands with pIs of 5.28 and 5.05 were observed for  
 25 PEG-CPG<sub>2</sub>. This shift towards acidic pI (see Figure 4) is indicative of the loss of basic amino groups on the protein when the lysine residues react with PEG. Hence the difference in pI between native CPG<sub>2</sub> and PEG-CPG<sub>2</sub> is proportional to the degree of PEGylation.

The apparent molecular size of PEG-CPG<sub>2</sub> was determined by polyacrylamide gel electrophoresis under reduced denaturing conditions (SDS-PAGE). PEG-CPG<sub>2</sub>, native CPG<sub>2</sub> and molecular weight markers  
 5 were analysed on a 4-20% pre-cast mini PAGE gel (Sigma). The native state CPG<sub>2</sub> is a homodimer of molecular weight 83,600 Da. Under SDS PAGE conditions the dimer dissociates into monomers of molecular weight 41,800 Da. From the molecular weight calibration curve (see  
 10 Figures 4 and 5) the apparent molecular size of the monomer unit of PEG-CPG<sub>2</sub> was determined to be 133,437 Da.

Similar preparations were made using PEGs of different molecular weights in the range 2000-9000 kDa.

#### 15 *Biodistribution*

Biodistribution studies were carried out in Nu/nu mice with or without human colon cancer xenografts LS174T as described in PCT/GB97/03284. These showed selective localisation in the tumour  
 20 xenograft compared with other tissues particularly after clearance of residual enzyme activity in blood was achieved with a monoclonal antibody (SB43) directed at the enzyme and galactosylated so as to clear rapidly from the blood *via* hepatic galactose receptors.

#### 25 *Immunogenicity of PEG CPG2*

Immunisation studies were performed in normal Balb/C mice using three preparations of PEG CPG2 given IP. The protocol for immunisation is shown in Table 1.

5

**Table 1: Immunisation schedule with mPEG-CPG2  
preparations (I) and (H)**

**A18 Protocol**

10

12 Balb c mice (3 groups x 4)

Group 1 CPG2-PEG "H"

Group 2 CPG2-PEG "I"

Group 3 native CPG2

15

17 OCT 97 25 µg CP compound to each mouse (ip in saline) as above

12 NOV 97 Test bleed

17 NOV 97 25 µg CP compound to each mouse (ip in saline) as above

26 NOV 97 Test bleed

5 JAN 98 25 µg CP compound to each mouse (ip in saline) as above

23 JAN 98 Test bleed

17 FEB 98 Group 1 split into 1A & 1B (each x2 mice)

Group 2 split into 2A & 2B (each x2 mice)

Group 3 maintained as a single group (x4 mice)

Groups 1A & 2A 25 µg CPG2-PEG5000 (ip in saline)

Groups 1B & 2B 25 µg native CPG2 (ip in saline)

Groups 3 25 µg native CPG2 (ip in saline)

24 MAR 98     Test bleed

CP = CPG2-PEG

Samples were tested by ELISA using methotrexate as substrate for CPG2  
5 and the results of the immunisation show that immunisation with native  
CPG2 produced an elevated anti-CPG2 response after the first  
immunisation. Preparation "H" (mPEG:CPG2 53:1. mPEG 2000 Da)  
produced a small antibody response in the ELISA after the second  
immunisation one month later. After a 3rd and 4th immunisation the  
10 ELISA response to H increased but even after the 4th immunisation was  
less than the controls after one immunisation with native CPG2. The four  
mice that received preparation "T" (mPEG:CPG2 = 60.1 mPEG 5000  
Da) showed no antibody response even after the 4th and 5th  
immunisations which were with native CPG2. Mice that received  
15 preparation NB (mPEG: CPG2 - 43:1 mPEG 5000 Da) had mixed  
responses. Some mice failed to respond when challenged with native  
CPG2 (tolerant), others responded with low levels of anti-CPG2 antibody.

The series of conjugates we have tested conjugated CPG2 with straight  
20 chain PEG using molecular weights of 2000 and 5000 with different  
amounts of PEG per molecule of CPG2. It was found that conjugates in  
which the number of PEG molecules per CPG2 molecule was 35 or less,  
were less immunogenic than native CPG2 in that the mouse antibody  
response to them occurred later than to the native enzyme. Branched  
25 chain mPEG may be advantageous compared with straight chain mPEG in  
terms of masking epitopes.

Conclusion:- Conjugates in which the average ratio of mPEG to CPG2 molecules, using PEG 5000 mol wt. was 60:1 were given at monthly intervals for three months to Balb/c mice by I.P. injection. Blood samples were taken 14 days after each injection. The blood samples were tested for interference of the reaction of CPG2 with methotrexate. No evidence of antibody activity was found in these samples. One month after the third injection of PEG-CPG2 two mice were challenged with native CPG2 and two with PEG-CPG2 (ratio 25:1) and again tested for antibodies to CPG2 after two weeks. No evidence of antibody production was found suggesting that immunological tolerance to CPG2 had been induced. This was confirmed by inoculating a native mouse with spleen cells from a tolerant mouse and naive mouse was then challenged with native CPG2 but it failed to generate an immune response thus confirming that immune tolerance had been induced.

15

Example 2: mPEG antibody-enzyme conjugates for prodrug activation and rescue agent deactivation at tumour sites

It has been shown that conjugates of methoxypolyethylene glycol and antibody enzyme conjugates (mPEG A5B7 CPG2) have more prolonged plasma half-lives than similar non-PEGylated conjugates (Eno-Amooquaye *et al*, 1996). The high level of PEG conjugate in blood is associated with greater concentration of enzyme accumulating at tumour sites than is achieved with the same antibody-enzyme conjugate (Figs 7 and 8). The PEG antibody enzyme conjugate also accumulates at tumour sites in greater amounts than PEG-enzyme conjugate when tested against the same xenografted tumour expressing the appropriate target antigen (Fig 9).

Although the slow clearance of PEG antibody-enzyme conjugate has been recognised (Amooquaye *et al* (1996) *Br. J. Cancer* 73, 1323-1327) there has been no reported attempt to exploit this potentially advantageous PEG-conjugate because tumour to blood ratios are less satisfactory than with  
 5 conventional antibody-enzyme conjugates which clear more rapidly from the blood.

Two factors come into play which make it possible to exploit the advantage of mPEG-antibody enzyme conjugates.

10

(1) The blood level of the conjugate carrying an appropriate amount of PEG can be reduced to undetectable level of enzyme activity by using an antibody directed at the enzyme or directed at the antibody. Normal tissue levels fall following the fall in plasma enzyme but enzyme is retained at  
 15 tumour sites.

(2) Antibody-enzyme conjugates to which a maximum or near maximum amount of mPEG is conjugated (ie all available lysine groups are PEGylated) is non-immunogenic and may be used to induce immune  
 20 tolerance. As a result repeated treatments may be given without invoking a host antibody response. The repeated treatments may use either non-PEGylated antibody enzyme or a PEGylated form of the conjugate where the amount of mPEG per molecule is optimised for tumour localisation and plasma clearance.

25

### Example 3

The high binding affinity of avidin and streptavidin for biotin has been explored in numerous studies. The general procedure has been to biotinylate anti-tumour antibodies which are radiolabelled and then to administer avidin or streptavidin as a clearing agent (Paganelli *et al* (1990) *J. Nucl. Med. Allied Sci.* 35, 88-89). The removal of the streptavidin-antibody conjugates from the blood is enhanced by galactosylating the avidin/streptavidin (Marshall *et al* (1995) *Br J. Cancer* 71, 18-24). The immunogenicity of avidin and streptavidin have been widely noted and limits their repeated use.

10

Attempts to control the immunogenicity of avidin/streptavidin have included the use of immunosuppressive agents, but the general immunosuppression these agents achieve is undesirable, their toxicity is significant and the delay in host response is limited.

15

An attempt was made to reduce the immunogenicity of streptavidin by conjugation to methoxypolyethylene glycol (Marshall *et al* (1995) *Br J. Cancer* 73, 565-572). The PEGylated galactosylated streptavidin was associated with lower antibody titres and in the number of mice that mounted an anti-gal streptavidin response. However, effective clearance of the radiolabelled biotinylated antibody was inhibited resulting in very little reduction in circulating radioactivity which was the objective of its use.

20

Although the mice which failed to mount an antibody response to PEG gal-streptavidin were challenged with non-PEG gal-streptavidin and failed to respond the significance of this was not appreciated and no further exploitation of immune tolerance was made.

25

It is clear that it would be advantageous if a state of immunological tolerance to avidin or streptavidin could be reliably induced prior to their use as a means of clearing radiolabelled biotinylated anti-tumour antibodies since this would allow the use of non-PEGylated avidin or streptavidin or galactosylated avidin or streptavidin which are known to be effective clearing agents.

To achieve immune tolerance to avidin and streptavidin it is necessary to ensure that all epitopes on the protein are masked. This is achieved by increasing the ratio of activated PEG molecules to avidin or streptavidin in the initial conjugation such that a further increase in the ratio results in no further increase in the PEG:protein ratio and thus to achieve the level of PEGylation necessary to reduce immune tolerance.

#### Example 4: Conjugation of dextran to CPG2

The conjugation of PEG or dextran to enzymes may be achieved by conventional techniques, for example as in WO 90/13540.

CPG2 may be coupled to soluble dextrans (Lomodex 40, Lomodex 70, Dextraven 110 and Dextraven 150, all trade marks; Fisons, Loughborough, Leics., UK) according to the method of Melton *et al* (1987). A volume of dextran preparation containing 1 g of dextran in 0.9% NaCl was diluted to 100 ml with 0.9% NaCl and reacted with cyanogen bromide (CNBr; Sigma, Poole, Dorset, UK). CNBr (0.5 g) was used for activating the 40- and 70,000 dalton dextrans and 0.4 g for the higher molecular weight dextrans. This reduction was necessary to



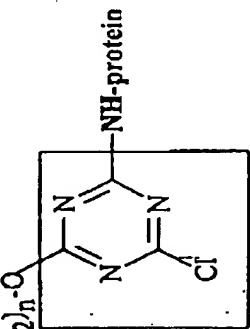
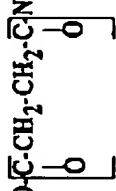
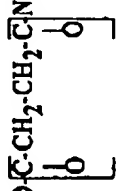



prevent precipitation of the 110,000 and 150,000 dalton dextrans. The reaction mixture was vigorously stirred at room temperature and maintained at pH  $10.7 \pm 0.1$  units in a pH-stat (Radiometer, Copenhagen, Denmark) by addition of 2 M NaOH. The CNBr was added as a finely divided powder in two equal portions at an interval of 20 min; the second portion was allowed to react until the pH of the reaction mixture was stable at 10.7; the pH was then adjusted to 9.0 and the mixture dialysed against running water for 2 hr at 4°C. The pH was brought back to 9.0 with 1 M NaOH and 1 ml enzyme solution (1265 U; 2.3 mg) in 0.1 M Tris-HCl buffer, pH 7.3, was added. The mixture was reacted overnight at 4°C after which 0.25 g glycine was added to block excess reactive sites. The mixture was stirred for a further 30 min and then concentrated to a volume of 40 ml in a model 202 concentrator using a PM10 ultrafiltration membrane (Amicon, Stonehouse, UK). The mixture (40 ml) was then chromatographed on a 1.3 litre bed volume of Sephadex G150 in a 4.4 x 87 cm column (Pharmacia, Uppsala, Sweden) and eluted with 0.05 M potassium phosphate buffer, pH 7.0. Fractions (10 ml) were collected and assayed for enzyme activity; carbohydrate content was determined by the phenol-sulphuric acid method (M. Dubois *et al* (1956) *Analyt. Chem.* 28, 350) using dextran-70 as standard in the range 0-100 µg/ml (Sephadex is a trade mark).

The peak fractions were pooled and concentrated to a volume of 10-12 ml as before. Enzyme activity and carbohydrate content were determined and protein content measured by the Coomassie blue method (M.M. Bradford (1976) *Analyt. Biochem.* 72, 248) using bovine serum albumin fraction V as standard in the range 0-100 µg/ml. The concentrated material was filter

sterilised (Millipore "Millex GS", 0.22  $\mu\text{m}$  pore size) and stored at  $-20^{\circ}\text{C}$ .

Millex GS is a trade mark.

Table 1.

METHOD	Coupling Moiety (boxed) and general structure of adduct	Bond	Co-product	reference
PEG-Cyanuric chloride	$\text{CH}_3(\text{-O-CH}_2\text{-CH}_2)_n\text{-O}$ 		HCl	(1,2,3,)
PEG-succinimidyl active ester ( succinimidyl succinate )	$\text{CH}_3(\text{-O-CH}_2\text{-CH}_2)_n\text{-O}$ 	amide bond ester bond	N-hydroxy-succinimide	(4,5,6)
PEG-succinate mixed anhydride	$\text{CH}_3(\text{-O-CH}_2\text{-CH}_2)_n\text{-O}$ 	amide bond ester bond	ester plus ester-modified protein	(7)
PEG-phenylchloroformates	$\text{CH}_3(\text{-O-CH}_2\text{-CH}_2)_n\text{-O}$ 	carbamate	substituted phenol	(8)
PEG-carbonyldiimidazole	$\text{CH}_3(\text{-O-CH}_2\text{-CH}_2)_n\text{-O}$ 	carbamate	imidazole	(9,10)
PEG-succinimide carbonate	$\text{CH}_3(\text{-O-CH}_2\text{-CH}_2)_n\text{-O}$ 	carbamate		(11)

Poly (PEG-MA) anhydride	$\begin{array}{c} \text{CH}_3(\text{-O-CH}_2\text{-CH}_2\text{)}_n\text{-O-CH}_2\text{-CH} \\   \\ \text{CH-COOH} \\   \\ \text{CH-CO-NH-protein} \\   \\ \text{CH}_2 \\   \\ \text{CH}_3(\text{-O-CH}_2\text{-CH}_2\text{)}_n\text{-O-CH}_2\text{-CH} \\   \\ \text{CH-COOH} \\   \\ \text{CH-COOH} \end{array}$	amide bond	(12)
PEG-maleimide	$\begin{array}{c} \text{CH}_3(\text{-O-CH}_2\text{-CH}_2\text{)}_n\text{NH-C(CH}_2\text{)}_5\text{-N} \\   \quad   \\ \text{O} \quad \text{O} \\ \text{S-protein} \end{array}$	amide bond	(13,14)
PEG-acetaldehyde	<p>no coupling moiety present or ethylene oxide unit</p> $\text{CH}_3(\text{-O-CH}_2\text{-CH}_2\text{)}_n\text{-O-CH}_2\text{-CH}_2\text{-NH-protein}$	secondary amine	(15)
PEG-sulphonic halogenide	<p>no coupling moiety present</p> $\text{CH}_3(\text{-O-CH}_2\text{-CH}_2\text{)}_n\text{-NH-protein}$	secondary amine	(16)

PEG-phenyl glyoxal				secondary amine		(17)
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## REFERENCES TO TABLE 1

- (1) Abuchowski *et al* (1977) *Journal of Biological Chemistry* 252, 3578-3581.
- 5 (2) Pyatak *et al* (1980) *Res. Commun. Chem. Pathol. Pharmacol.* 29, 113-127.
- (3) Yashimoto *et al* (1986) *Jpn. J. Can. Res.* 77, 1264-1270.
- 10 (4) Abuchowski *et al* (1984) *Cancer Biochem Biophys.* 7, 175-186.
- (5) Leonard *et al* (1984) *Tetrahedron* 40, 1585-1590.
- 15 (6) Katre *et al* (1987) *Proc. Natl. Acad. Sci. USA* 84, 1487-1491.
- (7) Wie *et al* (1981) *Int. Arch. Allergy. Appl Immunol.* 64, 84-99.
- (8) Veronese *et al* (1985) *Appl. Biochem. Biotechnol.* 11, 141-152.
- 20 (9) Beauchamp *et al* (1983) *Analytical Biochemistry* 131, 25-33.
- (10) Pizzo (1991) *Advanced Drug Delivery Reviews* 6, 153-166.
- 25 (11) Zalipsky (1985) WO 90/13540.

- (12) Yoshimoto *et al* (1987) *Biochemical and Biophysical Research Communications* **148**, 876-882.
- (13) Aldwin & Nitecki (1987) *Analytical Biochemistry* **164**, 494-501.
- (14) Goodson & Katre (1990) *Biotechnology* **8**, 343-346.
- (15) Harris *et al* (1989) *Applications in Cell Biology and Biotechnology* (pp 203-210) London: Plenum Press.
- (16) Delgado *et al* (1990) *Biotechnology and Applied Biochemistry* **12**, 119-128.
- (17) Maeda *et al* (1989) EPA 0 340741.

**CLAIMS**

1. A method of combating or diagnosing a tumour in a patient, the method comprising administering to the patient (a) an agent which  
5 tolerizes the patient to a said tumour selective agent or to an agent which interacts selectively with the said tumour selective agent; (b) a tumour selective agent which comprises a polypeptide; and (c) at least one further agent which interacts selectively with the said tumour selective agent.
- 10 2. A method according to Claim 1 wherein the tumour selective agent which comprises a polypeptide is an antibody-enzyme complex or an enzyme-macromolecule complex and the at least one further agent which interacts selectively with the tumour-selective agent is a relatively non-  
15 toxic prodrug which is converted into a cytotoxic drug by the enzyme, or is a rescue agent which is inactivated by the enzyme.
3. A method according to Claim 2 comprising administering a further agent which interacts selectively with the tumour selective agent which  
20 further agent is an inhibitor which reduces the level of active said enzyme at non-tumour sites, is an agent which accelerates the or clearance of the tumour selective agent from the blood.
4. A method according to Claim 3 wherein the inhibitor is an enzyme-inactivating small compound or an antibody.
- 25 5. A method according to Claim 3 wherein the clearance agent is an antibody which reacts with the enzyme.



6. A method according to Claim 1 wherein the tumour selective agent is an antibody-biotin complex and the at least one further agent which interacts selectively with the said tumour selective agent comprises avidin or streptavidin.

5

7. A method according to any one of Claims 1 to 5 wherein the agent in step (a) tolerizes the patient to the said tumour selective agent and comprises said polypeptide wherein immunogenic portions of the polypeptide are masked by a polymer.

10

8. A method according to Claim 6 wherein immunogenic portions of avidin or streptavidin are masked by a polymer.

9. A method according to Claim 7 or 8 wherein the polymer is mPEG.

15

10. A therapeutic system comprising (a) an agent which tolerizes a patient to a said tumour selective agent or to an agent which interacts selectively with the said tumour selective agent; (b) a tumour selective agent which comprises a polypeptide; and (c) at least one further agent which interacts selectively with the said tumour selective agent.

20

11. A kit of parts comprising (a) an agent which tolerizes a patient to a said tumour selective agent or to an agent which interacts selectively with the said tumour selective agent; (b) a tumour selective agent which comprises a polypeptide; and (c) at least one further agent which interacts selectively with the said tumour selective agent.

25

12. Use of an agent which tolerizes a patient to a tumour selective agent in the manufacture of an agent for combating or diagnosing a tumour in a patient wherein said tumour selective agent comprises a polypeptide and wherein said patient is administered said tolerizing agent prior to  
5 administration of said tumour selective agent and prior to administration of a further agent which interacts selectively with said tumour selective agent.
13. A polymer-enzyme conjugate wherein substantially all immunogenic  
10 sites of the enzyme are masked by the polymer and wherein the enzyme is able to convert a substantially inactive prodrug into a cytotoxic drug.
14. An antibody-enzyme-polymer conjugate wherein substantially all immunogenic sites of the enzyme are masked by the polymer and wherein  
15 the enzyme is able to convert a substantially inactive prodrug into a cytotoxic drug or wherein the enzyme is able to inactivate a rescue agent.
15. A conjugate according to Claim 13 or 14 wherein the polymer is monomethoxyl polyethylene glycol (mPEG).  
20
16. A conjugate according to any one of Claims 13 to 15 wherein the enzyme is selected from any one of hydrolases, amidases, sulphatases, lipases, glucuronidases, phosphatases or carboxypeptidases.
- 25 17. A conjugate according to any one of Claims 13 to 16 wherein the enzyme is carboxypeptidase G2.

18. A carboxypeptidase G2-monomethoxy polyethylene glycol conjugate consisting of at least 50 monomethoxy polyethylene glycol molecules per molecule of carboxypeptidase G2.

5 19. A method of destroying a target cell in a patient the method comprising administering to the patient (a) an agent which tolerizes the patient to a said target cell selective agent and (b) a target cell selective agent which comprises a target cell selective portion and an agent capable of killing the target cell.

10

20. A method according to Claim 19 wherein the agent capable of killing the target cell is a toxin.

15 21. A method according to Claim 20 wherein the toxin is any one of diphtheria toxin, ricin, abrin, gelonin, *Pseudomonas* exotoxin, an immunotoxin or OK-432.

20 22. A therapeutic system for treating a patient comprising (a) an agent which tolerizes the patient to a said target cell selective agent and (b) a target cell selective agent which comprises a target cell selective portion and an agent capable of killing the target cell.

25 23. Use of an agent which tolerizes a patient to a target cell selective agent in the manufacture of a medicament for destroying a target cell in the patient wherein the patient is administered said tolerizing agent prior to administration of said target cell selective agent.

24. A polymer-polypeptide conjugate wherein substantially all immunogenic sites of the polypeptide are masked by the polymer and wherein the polypeptide comprises a target cell selective portion and a portion capable of selectively killing the target cell.

5

25. A polymer-polypeptide conjugate according to Claim 24 wherein the polypeptide comprises a target cell selective antibody.

26. A polymer according to Claim 24 or 25 wherein the portion capable  
10 of selectively killing the target cell is a toxin.

27. Any novel therapeutic or diagnostic system as herein disclosed.

28. Any novel tolerogenic molecule as herein disclosed.

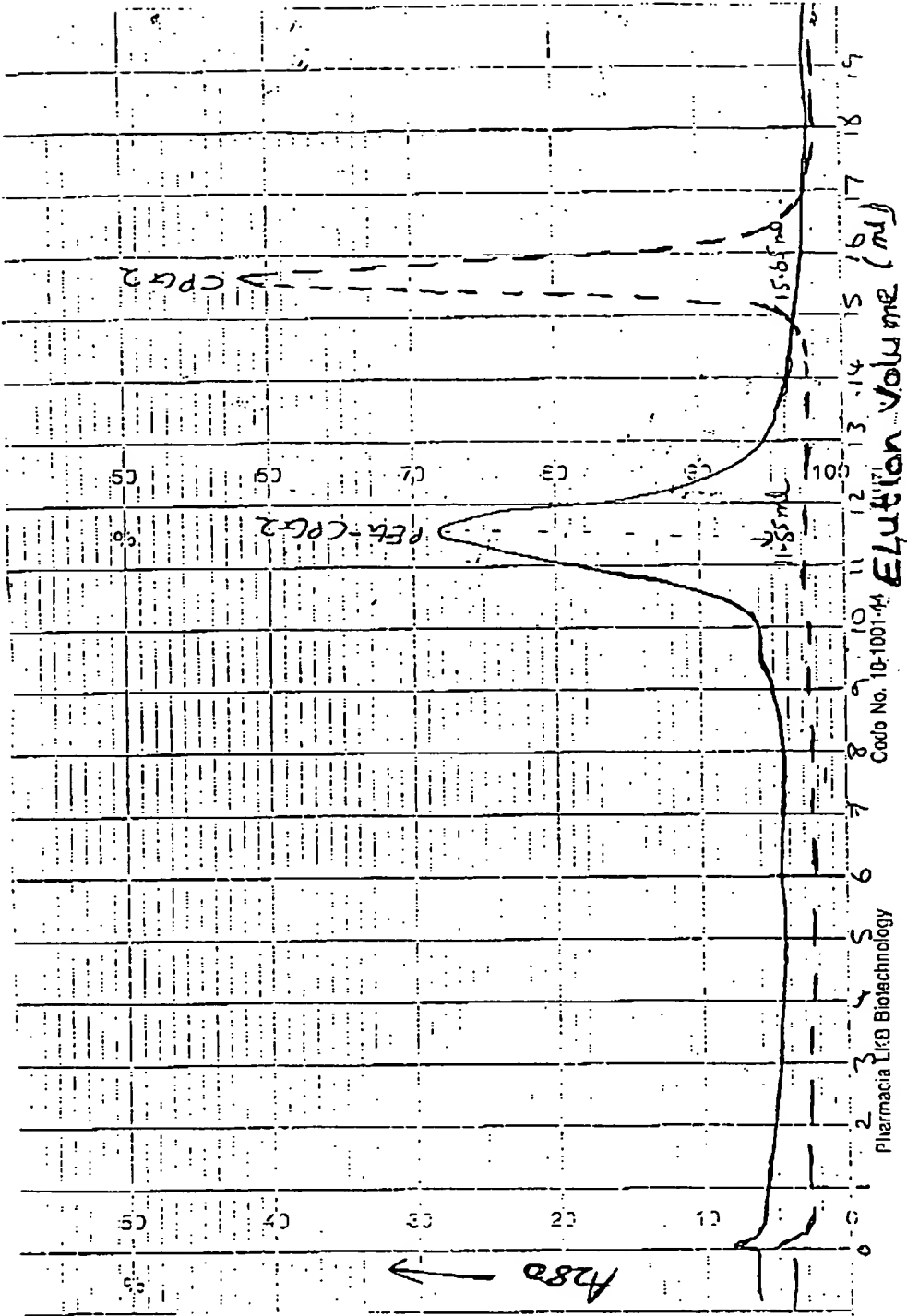
**ABSTRACT****TUMOUR THERAPY**

- 5 A method of combating a tumour in a patient, the method comprising administering to the patient (a) an agent which tolerizes the patient to a said tumour selective agent or to an agent which interacts selectively with the said tumour selective agent; (b) a tumour selective agent which comprises a polypeptide; and (c) at least one further agent which interacts  
10 selectively with the said tumour selective agent.

Figure 1

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Figure 1

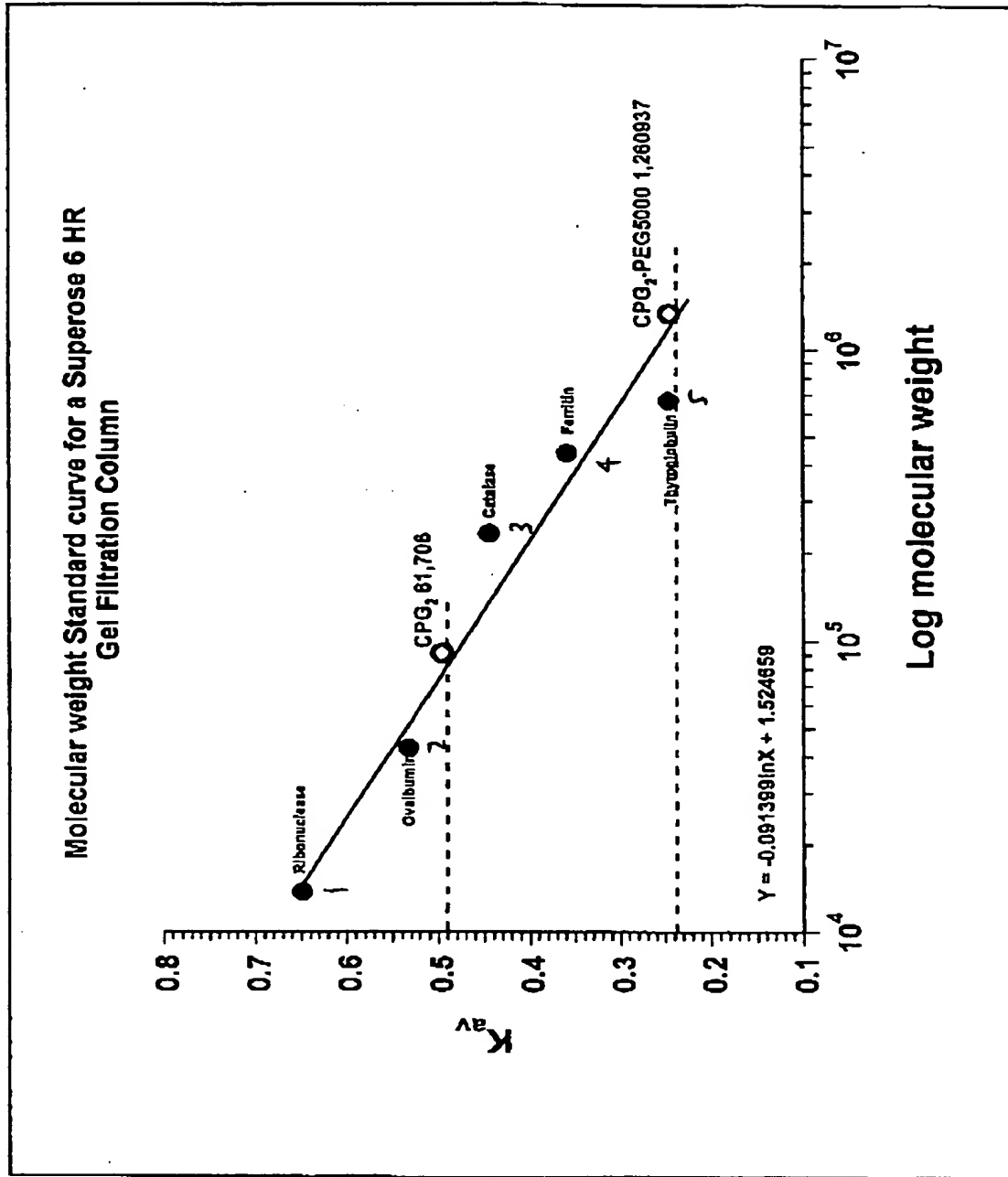


Superimposed gel filtration chromatogram of purified PEG-CPG2 and native CPG2 determined separately using a Pharmacia Superose 6HR 10/12 FPLC column. (—) PEG-CPG2 (---) native CPG2

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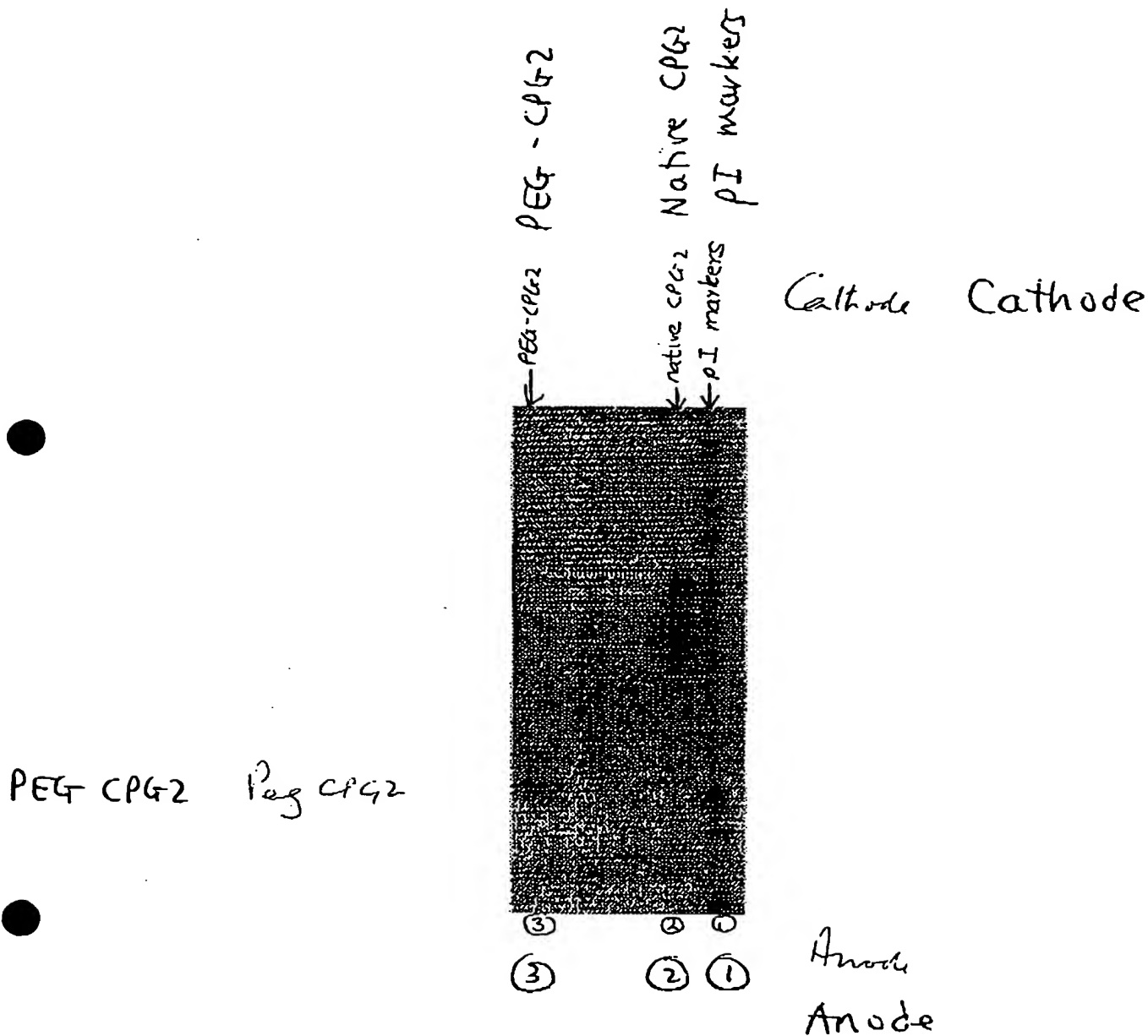
Figure 2



- 1 Ribonuclease
- 2 Ovalbumin
- 3 Catalase
- 4 Ferritin
- 5 Thyroglobulin

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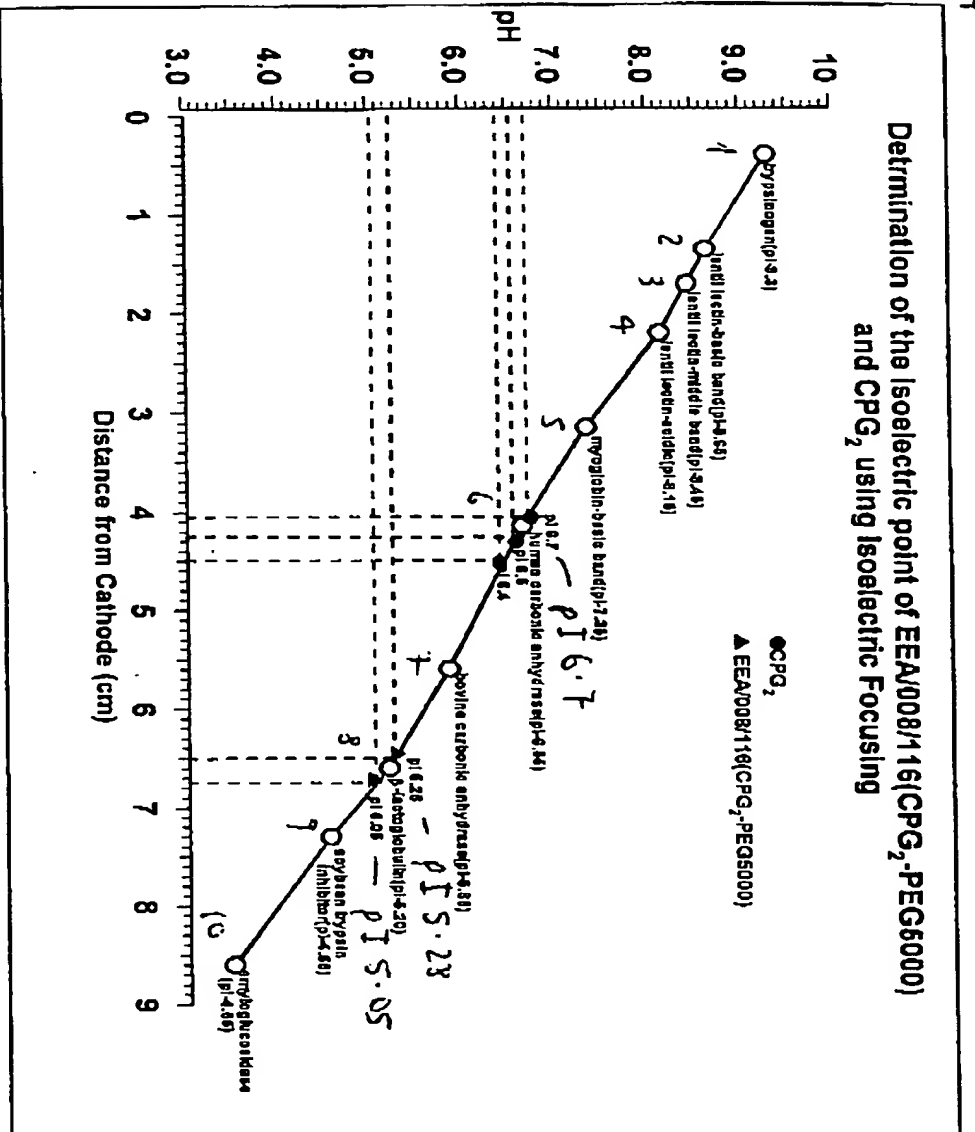
Figure 3 Isoelectric Focusing gel



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Figure 4



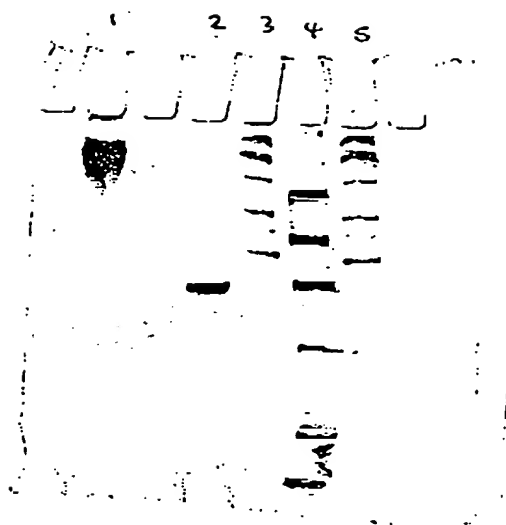
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- 1 tryptase (pI 9.3)
- 2 lentil lectin basic band (pI 8.59)
- 3 lentil lectin middle band (pI 8.49)
- 4 lentil lectin acidic band (pI 8.19)
- 5 myoglobin - basic band (pI 7.39)
- 6 human carbonic anhydrase (pI 6.55)
- 7 bovine carbonic anhydrase (pI 5.35)
- 8  $\beta$ -lactoglobulin (pI 5.20)
- 9 soybean trypsin inhibitor (pI 4.55)
- 10 amylglucosidase (pI 4.55)

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Figure 5

4-20% SDS-PAGE



- ① PEG-CPG2
- ② na'CPG2
- ③ High molecular wt markers
- ④ Low molecular wt markers
- ⑤ High molecular wt markers

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Figure 6

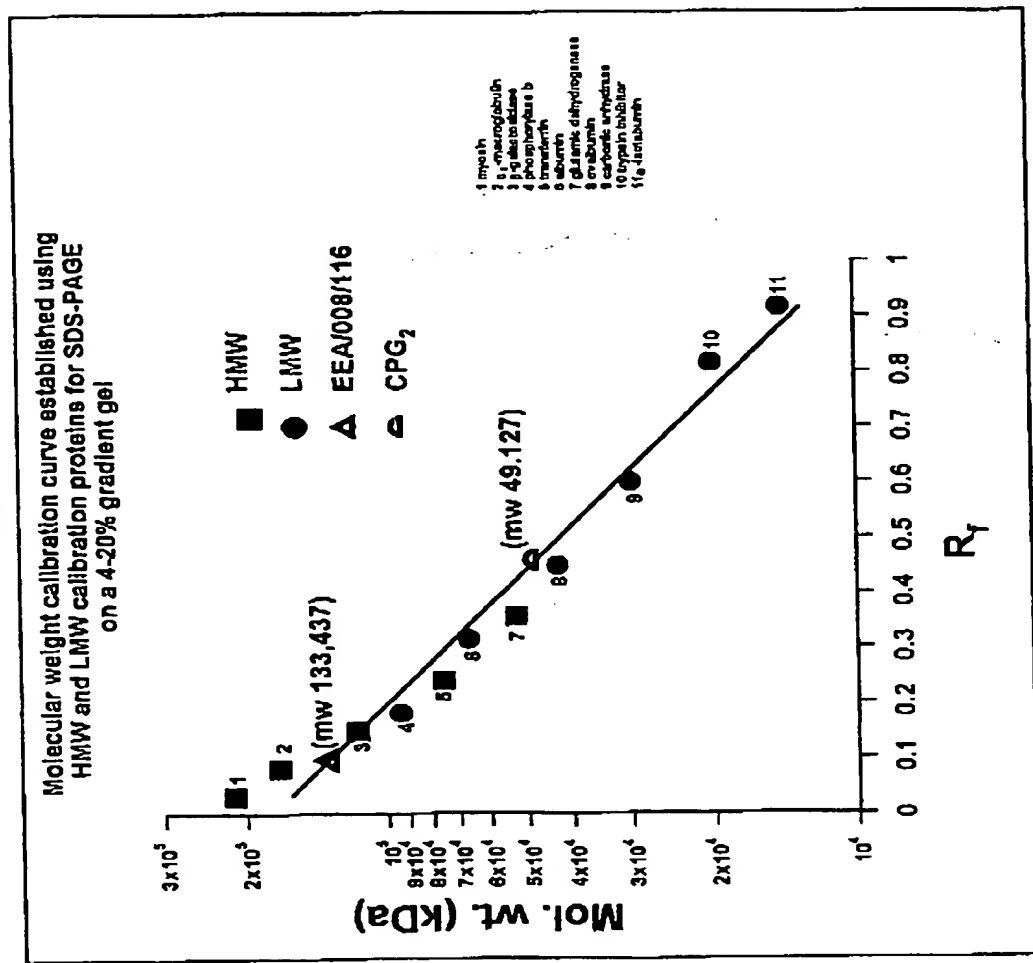
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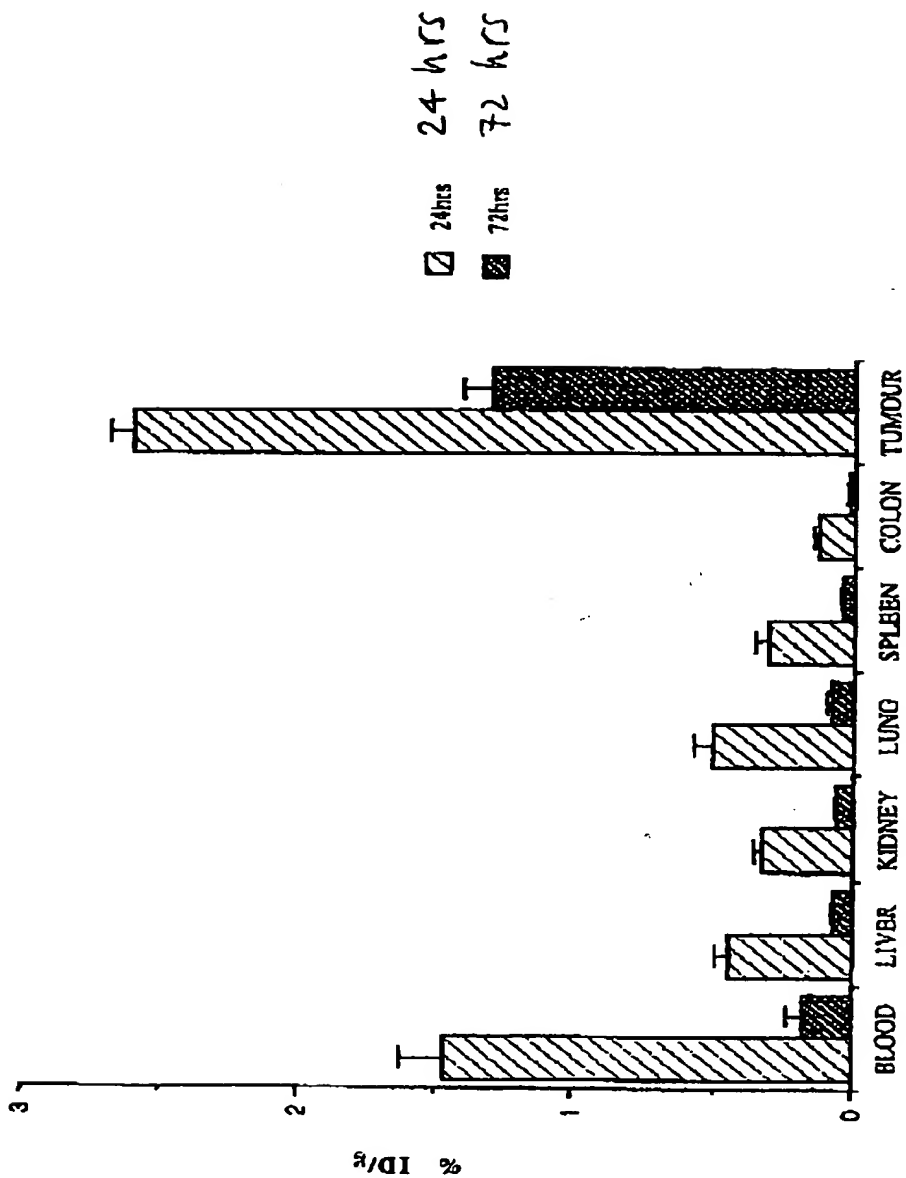
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# Blodistribution of 125-I-[ASB7-F(ab')2-CPG2] in LS174T xenografted nude mice.



% ID/g

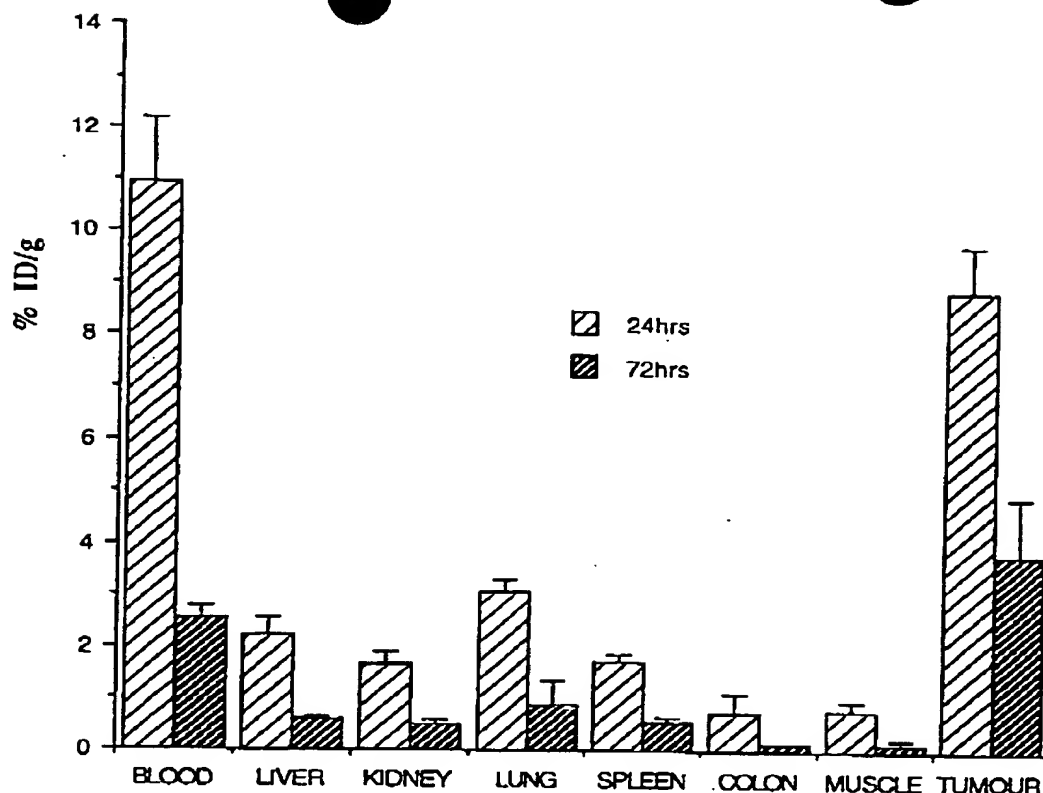
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Fig 7

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Biodistribution of  $^{125}\text{I}$ -[PEG-A5CP] in LS174 xenografts.

Fig 8



ASCP conjugate was PEGylated by Eburn at CXH. There are about 19-26 PEG molecules on the conjugate. Iodinated it at CXH, in med. conc. with 1mCi  $^{125}\text{I}$  iodide obtained from PWR Burke.

$^{125}\text{I}$ -ASCP was injected into LS174 bearing nude mice. Tissues collected at 24hrs and 72 hrs after injection for counts and also for autoradi.

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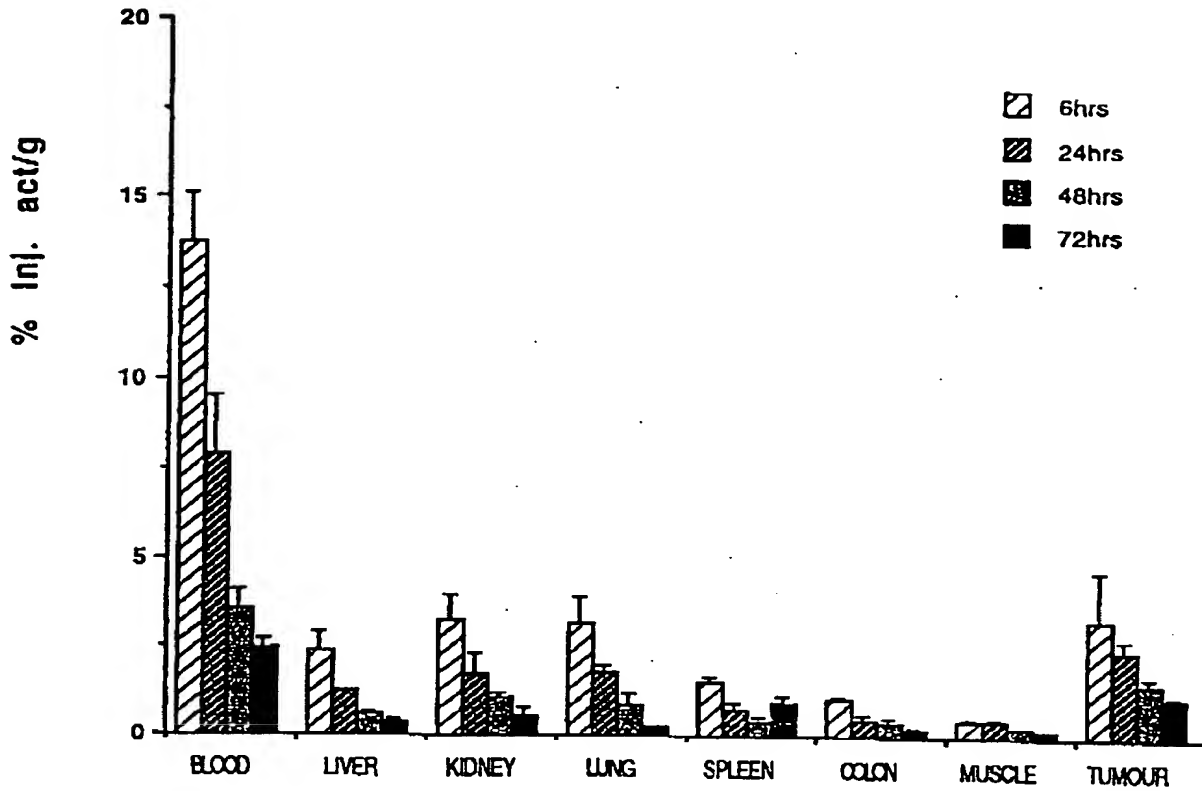
Fig 9

CPG2 - New Batch

43 kg/CPG2

SKS  
March 1998

**Biodistribution of <sup>125</sup>-Iodine labelled  
Peg-CPG2 in LS174T xenografted nude mice.**

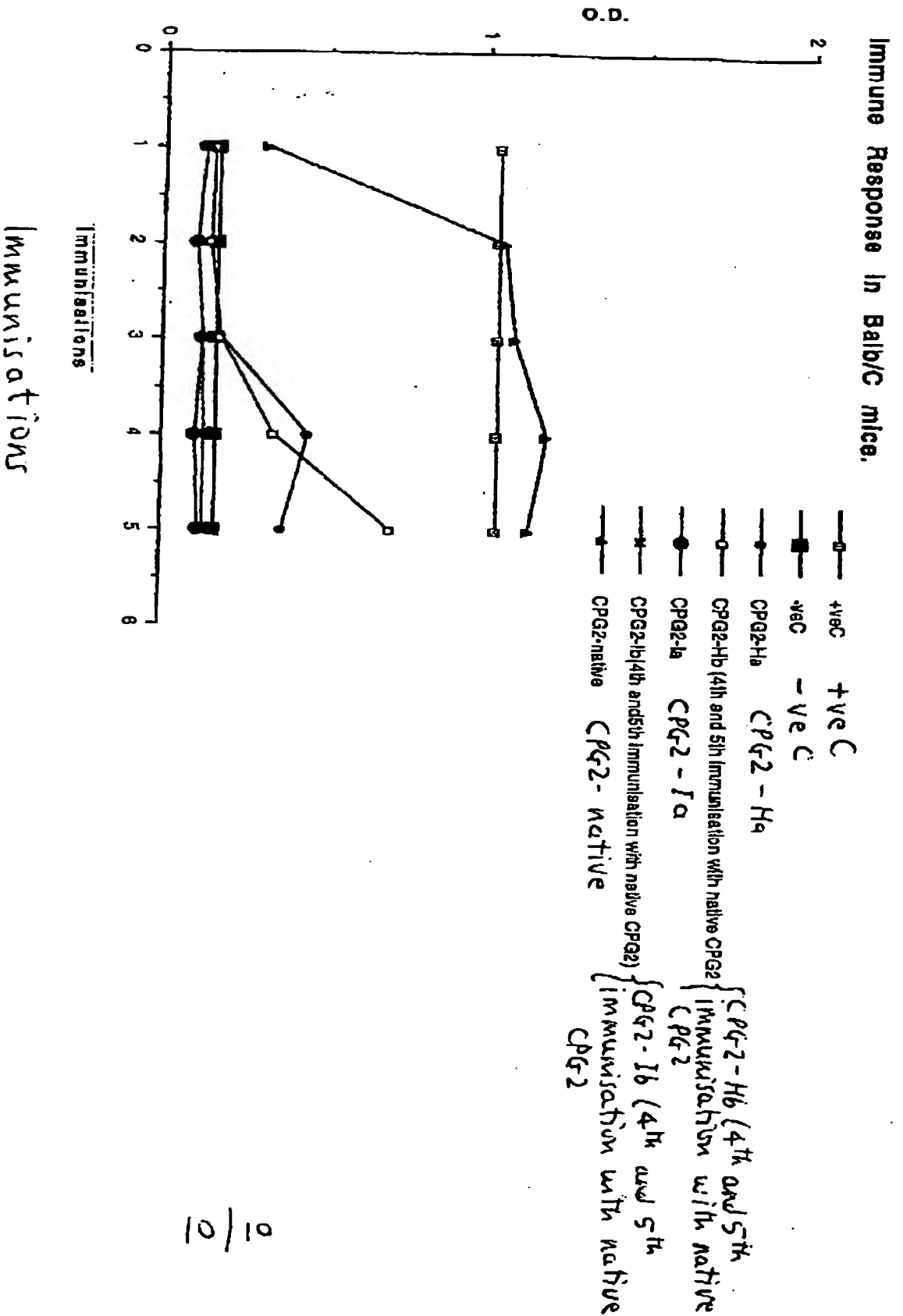


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Figure 10



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